Ph.D. thesis synopsis

Evaluation of Role of Secretory Phospholipase A2 in Lung Inflammation Elicited by Organic Dust

Mangalore University

Department of Studies & Research in Biochemistry

In fulfilment of the requirement for the degree of

Doctor of Philosophy in Biochemistry

Submitted by

Mr. Deepadarshan Urs

(Reg. No: MU/EXB/Ph.D-Reg./CR.72/Bio-Chem./2022-23/E.25)

Under the Guidance of

Dr. K.K. Dharmappa

Professor, Research Supervisor
Department of Studies & Research in Biochemistry
Mangalore University, Jnana Kaveri PG Centre,
Chikka Aluvara, Kodagu, India-571232

DEPARTMENT OF STUDIES & RESEARCH IN BIOCHEMISTRY

MANGALORE UNIVERSITY, JNANA KAVERI PG CENTRE, KODAGU

2021-2025

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INTRODUCTION

Inflammation is a necessary physiological response to injury and infections to combat pathogens and repair the damaged tissue (Hardy, 1989). Inflammation plays a vital role in all disease processes, including infections, trauma, sepsis, vascular pathology, and chemical and metabolic injuries. Tissue injury can be caused by external forces or internally disrupting chemical or biological substances. These injuries trigger inflammatory reactions, leading to dynamic and complex cytological and histological responses within and around the tissues.

Inflammation arises due to the physical and chemical activation of specialized sensory receptors known as nociceptors. These receptors detect potentially harmful stimuli and transmit signals to the brain, leading to the sensation of pain and impaired organ function. The host mounts a coordinated response to limit tissue injury and infection, facilitating tissue repair and functional recovery. This response includes the recruitment of immune cells and their migration to the site of injury, guided by signals from locally activated endothelial cells. Multiple organs participate in this complex reaction, including the central nervous system, which mediates fever; the cardiovascular system, which contributes through increased heart rate; the liver, which produces acute-phase plasma proteins; and the endocrine system, which modulates the process through adrenal and other pathways. Inflammation may be presented as an acute, short-term reaction or persist as a chronic, long-lasting condition.

Historical Aspects of Inflammation

Inflammation is a comprehensive phrase that was first introduced by Aulus Celsus prior to 45 AD. The body's response to stimuli is characterized by four primary signs: redness, heat, swelling, and pain (Celsus AC 1961; Cavaillon, 2021). Approximately two centuries later, Galen described the humoral theory of inflammation. According to Galen, the generation of pus was a constructive component of the body's response to injury rather than a secondary pathological event. This humoral process of inflammation was believed to be the proven theory until the nineteenth century. Further, Virchow in 1871 introduced the fifth cardinal sign of inflammation - *functio laesa* (loss of function). Unlike Galen, Virchow regarded inflammation as an intrinsically pathological process (Virchow R, 1871).

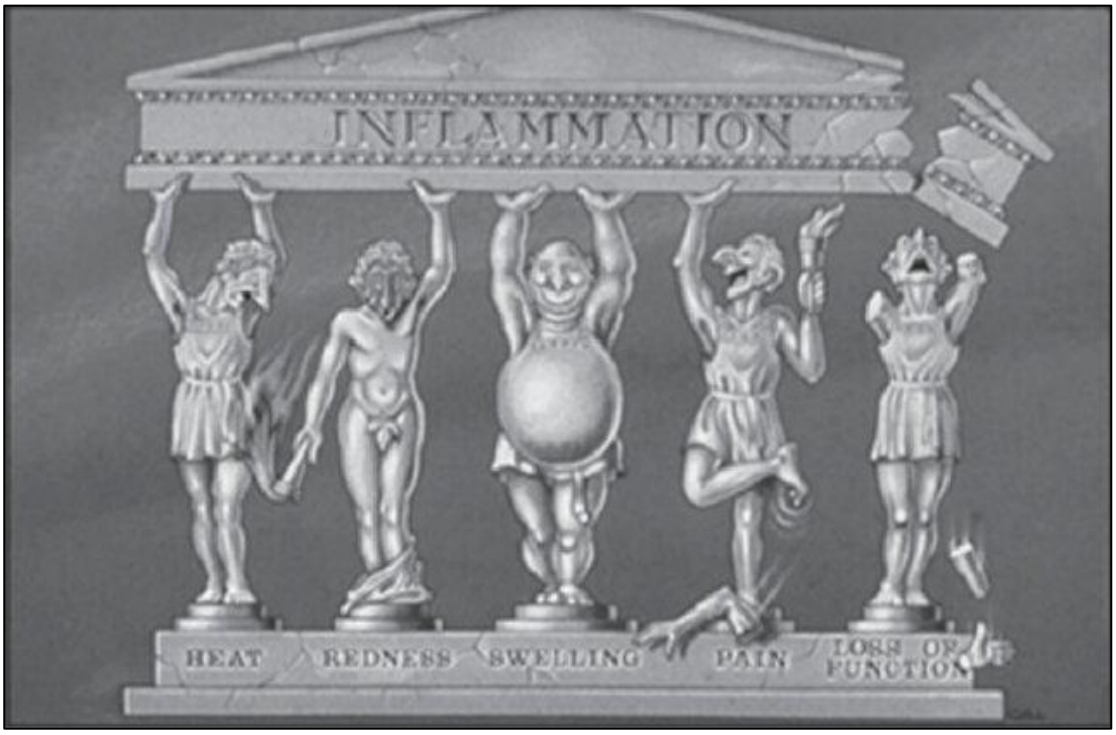


Figure 1.01: Signs of inflammation

Types of inflammation

The inflammatory reaction to tissue injury and external stimuli ultimately results in the reinstitution of tissue structure and function. This reaction necessitates the activation of innate immunity in certain instances and an adaptive immunological response in others. The two foremost essential components of the host's defence mechanism are these reactions. In addition to serving as the initial barrier against harmful substances, innate immunity also transmits the required signals to direct the adaptive immune system to initiate a reaction (Clark R 2005). Furthermore, the adaptive immune system depends on the innate immune system to supply the essential effectors, such as phagocytes and granulocytes, to address the triggering event (Trowbridge and Emling, 1997; Abbas, 2003). Based on this perspective, inflammatory responses are primarily classified as acute inflammation and chronic inflammation.

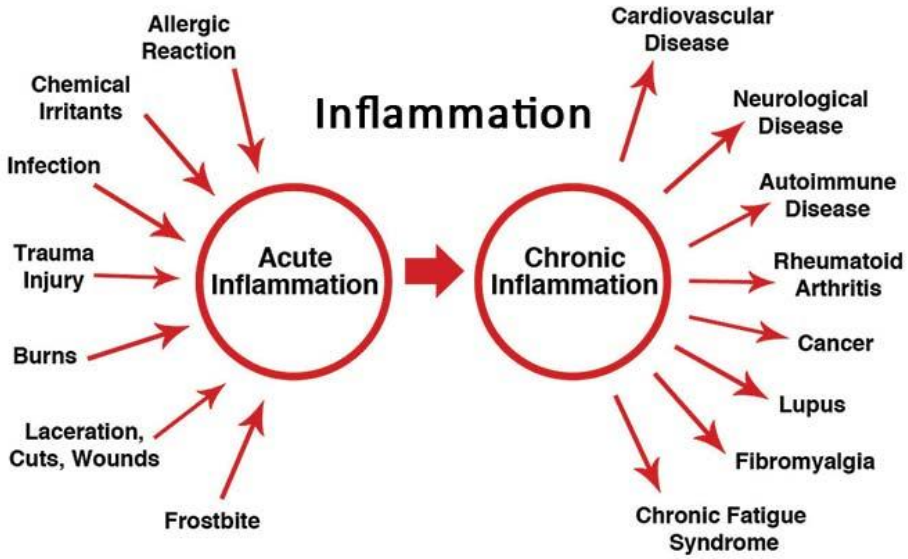


Figure 1.2: This figure illustrates the progression from acute inflammation, triggered by various external factors like infections and injuries, to chronic inflammation, which is a key contributor to numerous long-term diseases, including cardiovascular disease, autoimmune disorders, and cancer. It demonstrates how the body's short-term protective response, when left unresolved, can lead to a sustained and damaging state of inflammation.

Acute inflammatory reactions

Acute inflammation may begin within minutes or hours, contingent upon the nature and extent of the tissue injury and often persists for hours to days (Ward PA 1989). If the patient survives acute inflammation, there are four primary outcomes: (i) resolution, (ii) healing by fibrosis, (iii) abscess formation, and (iv) progression to chronic inflammation in certain circumstances (e.g. virus invasions, foreign body reactions, and fungal infections). Three variables influence these results: the extent of tissue injury, the ability of specialized cells in the injured tissue to undergo division and replacement, known as regeneration, and the specific agent responsible for the tissue damage (Lawrence T 2002). Although acute inflammation can be resolved or healed by scarring, it can also advance to chronic inflammation, and it is typical for a combination of acute and chronic responses to occur simultaneously (Lawrence et al., 2002).

Chronic inflammatory reactions

If an inflammatory agent persists for an extended period, chronic inflammation may be succeeded by acute inflammation, resulting in simultaneous tissue damage and inflammation. Chronic inflammation is defined by the rapid growth of blood vessels and connective tissue, accompanied by an elevated level of lymphocytes and macrophages (Trowbridge HO 1990). In rheumatoid arthritis and other chronic inflammatory disorders are distinguished by the excessive buildup and long-lasting presence of inflammatory cells in synovial joints, leading to joint deterioration (Scott DL 2007). Additional disorders, including chronic

bronchitis, emphysema, asthma, glomerulonephritis, myocardial infarction, and ischaemia reperfusion damage, also exhibit tissue or organ dysfunction due to an improper inflammatory response (Abbott, 2000; Ansar W 2016; Lawrence et al., 2002). The extensive range of potentially fatal consequences underscores the need to manage chronic inflammatory diseases.

Mechanisms of lung inflammation

Inflammation in the lungs is a complex, multi-phase physiological response to injury, infection, or the presence of harmful particles. It involves tightly regulated interactions between resident structural cells, infiltrating immune cells, and a dynamic network of soluble mediators including cytokines, chemokines, lipid derivatives, and proteolytic enzymes (Moldoveanu B, et al., 2009). While inflammation is a necessary defense mechanism, its dysregulation or chronic persistence contributes to pathogenesis in a range of pulmonary diseases such as asthma, chronic obstructive pulmonary disease (COPD), interstitial lung diseases, and environmentally induced pneumonitis (Bringardner BD et al., 2008). Understanding the fundamental mechanisms that govern lung inflammation is crucial for identifying specific molecular targets relevant to occupational exposures, such as those seen with organic dust in poultry farming environments.

Chronic lung inflammation and lung pathologies

Chronic inflammation is considered one of the central pathological processes that contributes to the initiation and progression of many lung diseases, and over the years our understanding of how persistent inflammatory responses shape pulmonary structure and function has evolved considerably, yet it still

remains a subject of active investigation because the lung is constantly exposed to external insults and environmental irritants that make it particularly vulnerable to ongoing injury and repair imbalance, and when inflammation becomes unresolved it gradually develops into a condition that not only affects the resident immune cells but also alters the epithelial, endothelial, and stromal compartments of the respiratory system (Lee, G 2009).

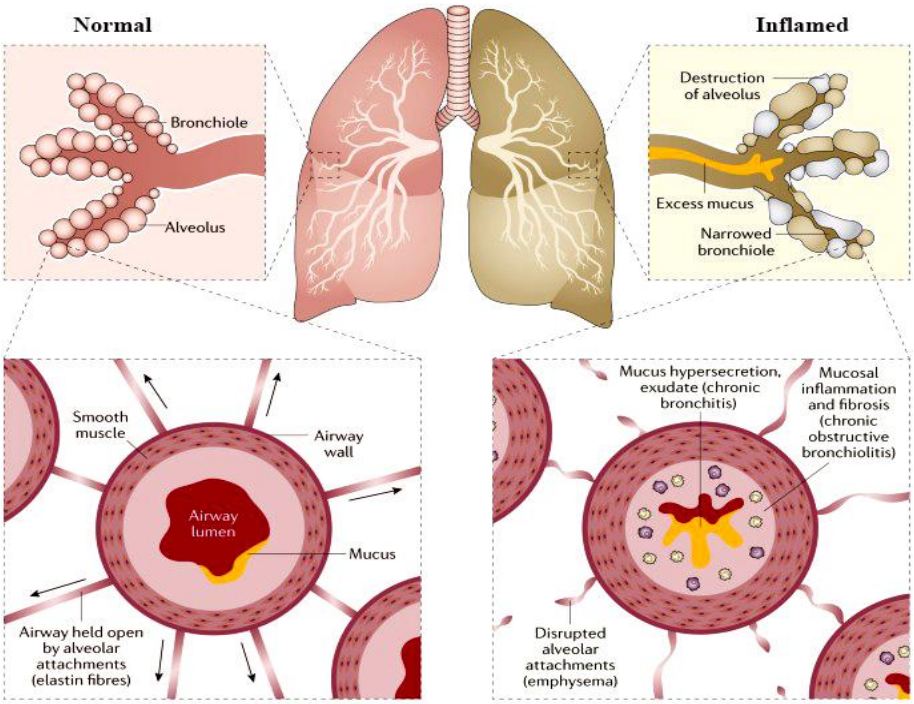


Figure: The figure illustrates the "Normal vs. Inflamed Lungs," visually comparing a healthy respiratory system with one impacted by chronic lung disease.

Occupational Lung Diseases and Organic Dust Exposure

Agricultural and industrial exposures represent important risk factors for chronic lung inflammation and pathologies, because the lungs are the first organs to interact with airborne particles, gases, vapors, and organic

materials generated in farming activities, livestock operations, construction, and different manufacturing industries, and over time this constant exposure contributes to the initiation of persistent immune responses that fail to resolve, gradually leading to irreversible lung damage (Faria NM 2006; Murgia N, 2020; Schwab AD et al., 2024).

Composition and physicochemical nature of poultry organic dust

The composition and physicochemical nature of poultry organic dust is highly complex because it arises from a mixture of materials generated within poultry housing environments, including feathers, fecal matter, litter, feed particles, skin debris, bedding material, microorganisms, and their by-products, all of which combine to create an airborne particulate mixture with significant biological activity (Bindari, Y. R., et al., 2021; Gržinić G, et al., 2023). Unlike inorganic dust that is chemically uniform, poultry dust is heterogeneous and dynamic, changing with the type of production system, animal density, ventilation, bedding substrate, feed composition, and management practices, and this variability makes it difficult to define a single chemical profile.

Although a considerable number of studies have explored the respiratory outcomes associated with poultry organic dust exposure and the mechanisms underlying inflammation through endotoxins, β-glucans, ammonia, oxidative stress, and innate immune signalling, there remains a significant gap in understanding the role of specific lipid mediators and their regulatory enzymes in this process, particularly secretory phospholipase A_2 ($sPLA_2$). Most of the experimental and occupational investigations have

focused on cytokines, chemokines, and pattern recognition receptor pathways, while the contribution of phospholipid metabolism to the inflammatory cascade has received limited attention. sPLA2 is known to hydrolyze membrane phospholipids to release arachidonic acid and lysophospholipids, which are precursors for eicosanoids and other potent bioactive lipids that orchestrate inflammatory responses. In many chronic inflammatory lung conditions such as asthma, COPD, and acute lung injury, sPLA2 has been implicated in amplifying inflammation by regulating leukotriene and prostaglandin synthesis, enhancing vascular permeability, and modulating immune cell activation. However, in the context of poultry dust exposure, there is little direct evidence demonstrating whether sPLA2 is activated, to what extent it contributes to neutrophilic influx or epithelial injury, and how it interacts with other signalling pathways such as TLR4, NF-κB, or MAPKs.

Background of Secretory phospholipase A2

Secretory phospholipase A2, generally called sPLA2, belongs to a large family of phospholipase A2 enzymes that hydrolyze the sn-2 ester bond of membrane glycerophospholipids, releasing free fatty acids such as arachidonic acid and lysophospholipids, both of which are important precursors for bioactive lipid mediators. Unlike cytosolic PLA2s that are larger and function inside cells, sPLA2s are relatively small, extracellular enzymes that are secreted into biological fluids, act in the extracellular space, and rely on calcium ions for catalytic activity. Structurally, sPLA2s are compact proteins of about 120–150 amino acids with a molecular weight typically between 13–19 kDa. They

contain a well-conserved calcium-binding loop with the consensus sequence YGxG, which stabilizes substrate binding and catalytic transition states. The catalytic machinery is centered on a histidine–aspartate dyad, with histidine acting as a general base to activate a water molecule, enabling nucleophilic attack on the sn-2 ester bond. Another structural hallmark is the presence of multiple disulfide bonds, often 6–8, which provide strong conformational stability against proteolysis, temperature changes, and oxidative environments, explaining why these enzymes remain active in diverse extracellular conditions such as plasma, synovial fluid, or airway secretions.

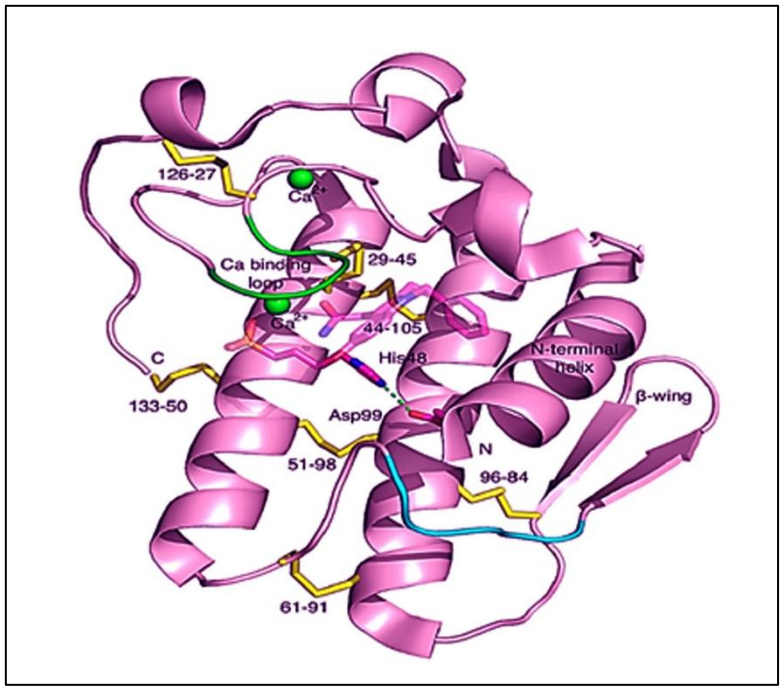


Figure: The figure illustrates the three-dimensional structure of phospholipase A2. This enzyme is presented in a ribbon representation, showcasing the arrangement of its helices and beta-strands. Key active site residues, including histidine and aspartate, are highlighted to illustrate their role in substrate interaction and catalysis. The structural model highlights the enzyme's hydrophobic funnel, which is crucial for substrate binding and specificity (Kim, R.R et al., 2020).

Isoform of Secretory phospholipase A2

Isoform diversity is a defining feature of sPLA2 biology. In humans, more than ten functional isoforms have been described, each encoded by distinct genes and showing variations in amino acid sequence, tissue expression, and biological role. These include group IB (PLA2G1B), IIA (PLA2G2A), IIC, IID, IIE, IIF, III (PLA2G3), V (PLA2G5), X (PLA2G10), and XII (PLA2G12A and PLA2G12B) (Murakami M et al., 2016).

$sPLA_2$-IIA: regulation, secretion, and downstream lipid mediator synthesis

Secretory phospholipase A_2 group IIA ($sPLA_2$-IIA) has emerged as one of the most biologically significant members of the phospholipase A_2 superfamily because of its dual ability to function as a host defense molecule and as a potent amplifier of inflammatory responses through the liberation of lipid mediators, and for this reason the regulation, secretion, and downstream pathways of $sPLA_2$-IIA have been studied extensively in the context of acute and chronic inflammatory diseases, particularly those affecting the lung (Hurley BP 2008). The expression of $sPLA_2$-IIA is usually minimal under physiological conditions, but during inflammation, it is dramatically upregulated, reflecting its role as an inducible enzyme designed to respond to cellular stress, microbial invasion, and pro-inflammatory signaling. The regulation occurs primarily at the transcriptional level, where cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) strongly activate $sPLA_2$-IIA transcription through nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), and signal transducer and

activator of transcription (STAT) binding to promoter regions (Murakami 2016; Sullivan CP, et al., 2010).

sPLA$_2$-IIA Inhibitors as therapeutic agents for pulmonary inflammation

Quercetin was the first flavonoid identified as a PLA$_2$ inhibitor, displaying activity against secretory PLA2 (sPLA2) derived from various inflammatory sources (Lee et al., 1982). Among flavonols, compounds such as kaempferol, quercetin, and myricetin exhibit strong inhibitory effects on snake venom PLA2.

Over the past decade, approximately 40 to 50 inhibitors of secretory phospholipase A$_2$ (sPLA$_2$) have been identified, and this number continues to expand. Despite this progress, only a limited subset shows meaningful potential for clinical application. Most promising candidates belong to the class of active site–directed inhibitors, including compounds such as BMS-181162, LY311727, ARL-67974, FPL67047, SB-203347, Ro-23-9358, YM-26734, and IS-741. Structural analogs of YM-26734 have also been synthesized and demonstrate potent inhibitory activity at nanomolar concentrations. Pharmaceutical interest in this field remains strong, with companies such as Eli Lilly and Shionogi advancing agents like LY-315920 and PIP-18 for the treatment of sepsis and other inflammatory disorders (Thwin et al., 2009; Thwin et al., 2010). Notably, several highly potent, selective, and bioavailable inhibitors of human sPLA$_2$ group IIA have been available for more than a decade, providing robust experimental evidence for a pathogenic role of sPLA$_2$ group IIA in diverse animal models of inflammatory disease.

From our research group we identified many sPLA2-IIA inhibitors such as oleanolic acid (Dharmappa et al., 2010), Genistein (Dharmappa et al., 2009), Tricyclic Dipyrido Diazipinone derivatives 6(a-f) (Timmegowda et al., 2007), Ascorbic derivatives (Riyaz M et al., 2010) and very recently we identified Elemolic acid (Giresha A.S et al., 2022), Sinapic acid (Giresha A.S et al., 2022), and Quercitrin (Sophiya P et al., 2023), Corosolic acid (Pundalik S et al., 2023). Moreover, these inhibitors irreversibly bind to sPLA2 enzyme and inhibits its activity, as demonstrated by *in vitro*, *in situ*, and *in vivo* experiments. Research on the inhibitory mechanism indicates that the inhibition of $sPLA_2$ is independent of the concentration of calcium or substrate. Modified intrinsic fluorescence and significantly diminished negative bands in the CD spectrum indicate direct interaction between the inhibitor and the sPLA2 enzyme.

Hence in the current study we investigate the anti-inflammatory function of the naturally occurring $sPLA_2$-IIA inhibitor oleanolic acid and synthetic sPLA2 inhibitor diazepinone derivative 6f for elucidating the cellular and molecular mechanisms in poultry organic dust induced inflammation is essential for understanding the onset of lung diseases. This entire investigation provides insights into lung etiology from an alternative perspective and underscores the significance of inhibitors and that helps to understand mechanism of $sPLA_2$-IIA mediated induction of lung inflammation.

AIM AND OBJECTIVES

- Determine whether naturally occurring and synthetic $sPLA_2$ inhibitors suppress the induction of inflammatory mediators by organic dust extract in Beas2B and primary normal human bronchial epithelial cells.

- Determine whether induction of inflammatory mediators by organic dust in lung epithelial cells is associated with
    - Activation and/or increased expression of cPLA2, sPLA2, mitogen-activated protein kinases (MAPKs), and COX-2.
    - Increased formation of arachidonic acid metabolites such as prostaglandin E2 (PGE2) and thromboxane B2 (TXB2).

- Determine whether naturally occurring and synthetic sPLA2 inhibitors suppress the induction of lung inflammatory mediators and lung infiltration of inflammatory cells by organic dust extract in mice.

MATERIALS AND METHODS

1. **Chemical Reagents:** Oleanolic acid (≥97% purity) was procured from Sigma Aldrich. Cell culture reagents including Ham's F12 medium, fetal bovine serum, Penicillin-Streptomycin, and Amphotericin B were obtained from Thermo Fisher Invitrogen. All chemicals used were of analytical grade purity.

   Assay Kits: Secretory phospholipase A2 assay kit was obtained from Cayman Chemicals. ELISA kits for prostaglandin E2 and thromboxane B2 (both human and mouse) were procured from Krishgen Biosystems.

   Cytokines and Chemokines: Human and mouse cytokines (IL-6, IL-8, TNF-α) and chemokines (MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5) were purchased from Preprotech Asia and Krishgen Biosystems.

   Primary Antibodies: $sPLA_2$-IIA antibody was obtained from Santa Cruz Biotechnology. Antibodies for cPLA2, p-cPLA2, ERK1/2, p-ERK1/2, P38, p-P38, and β-Actin were purchased from Cell Signaling Technology. Secondary HRP-conjugated antibodies were from Thermofisher Scientific.

2. **Analytical Instruments:** NMR spectra were recorded on JNM-ECZ500R/S1 (500 MHz) spectrometer. HRMS analysis was performed on Xevo G2-XS Q-Tof mass spectrometer. HPLC analysis utilized Sunfire C18 column on Waters 2545 Binary Gradient Module.

3. **Compound Synthesis** Target Compound: 11-cyclopropyl-5H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6(11H)-one was synthesized through a three-step process involving nucleophilic substitution, cyclization, and purification by column chromatography. The final compound was characterized by ^{1}H NMR, ^{13}C NMR, HRMS, and HPLC with 99.85% purity.

4. **Computational Studies**

   Molecular Docking: AutoDock Vina was used with Phospholipase A2 crystal structure (PDB: 1POE). Varespladib served as reference ligand. Protein preparation involved removal of water molecules and addition of hydrogen atoms using Discovery Studio and MGL Tools.

   Molecular Dynamics: 100 ns MD simulation was performed in GROMACS with SPCE water model and physiological salt concentration (0.15 M NaCl). Analysis included RMSD, RMSF, radius of gyration, and binding free energy calculations using MM-PBSA method.

5. **Dust Extract Preparation**

   Poultry dust collected from broiler farms in Kodagu, Karnataka was processed by incubating with F12K media (1:10 ratio) containing antibiotics. The suspension underwent ultrasonic treatment, centrifugation (800g and 10,000g), filtration through 0.2-µm filter, and storage at -20°C.

6. **Cell Culture Studies**

   Cell Lines: A549 lung epithelial cells were obtained from NCCS, Pune. Cells were maintained in Ham's F-12K medium with 10% FBS and antibiotics under standard culture conditions.

Cytotoxicity Assessment: MTT assay was performed using standard protocol. Cells were treated with test compounds for 48 hours, followed by MTT incubation and absorbance measurement at 570 nm.

Treatment Protocol: Cells underwent 2-4 hours serum starvation before treatment with dust extract and oleanolic acid. Post-treatment, culture media was collected, and cells were lysed for protein extraction.

7. **Animal Studies**

Ethics and Animals: All procedures were approved by Institutional Animal Ethics Committee, Mangalore University (MU/CARRT/IAECMU-RP-15/15/2022-23). BALB/c mice (8 weeks, 20-25g) were housed under standard conditions with 12-hour light/dark cycle.

Exposure Model: Mice received daily intranasal administration of either sterile PBS or 20% dust extract (50 µL) for 4 weeks to simulate occupational exposure. Animals were euthanized 5 hours post-final exposure.

Sample Collection: Blood was collected for serum separation. Bronchoalveolar lavage fluid (BALF) was obtained using 3×1 mL PBS washes. Lung tissues were processed for histological, biochemical, and molecular analyses.

8. **$sPLA_2$ Activity:** Measured using the Cayman Chemical kit with spectrophotometric detection at 414 nm. Activity calculated as µmol/min/mL using standard formula.
9. **COX-2 Activity:** Determined using Cayman COX assay kit with arachidonic acid substrate and colorimetric detection at 590 nm.

10. **BALF Analysis:** Total and differential cell counts performed using a hemocytometer and Giemsa staining. Protein content estimated by Lowry's method.

11. **Inflammatory Mediators:** Cytokines (TNF-α, IL-6, IL-8) and chemokines (MCP-1, MIP-1α, RANTES) quantified by ELISA. Prostaglandin E2 and thromboxane B2 levels measured using specific ELISA kits.

12. **RNA Isolation:** Total RNA extracted from cells and lung tissues using Aurum Total RNA Mini Kit (Bio-Rad) with DNase treatment. RNA quality assessed by spectrophotometry.

13. **qRT-PCR:** Gene expression analysis performed for PLA2G2A using specific primers. GAPDH served as housekeeping gene for normalization.

14. **Western Blotting:** Protein expression of $sPLA_2$-IIA, cPLA2, phosphorylated forms, and MAPK pathway proteins analyzed. Membranes probed with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies and TMB detection.

15. Histological Analysis

Tissue Processing: Lung tissues fixed in 10% formalin, processed through graded alcohols, embedded in paraffin, and sectioned at 5 µm thickness.

H&E Staining: Standard hematoxylin and eosin staining performed for morphological assessment. Pathological changes scored (0-3 scale) by blinded investigators.

Immunohistochemistry: $sPLA_2$-IIA expression detected using specific antibody with DAB chromogen and hematoxylin counterstaining. Sections processed using commercial IHC kit.

16. **Lung Function Parameters:** Wet-to-dry weight ratios and lung-to-body weight ratios calculated to assess pulmonary edema and inflammation.

17. Statistical Analysis

Data analysis performed using GraphPad Prism 10.2.3. Statistical significance determined by two-tailed Student's t-test or ANOVA with Dunnett's multiple comparisons test. Results expressed as mean ± SEM with $p<0.05$ considered statistically significant. All experiments performed with appropriate sample sizes and technical replicates.

RESULTS

Organic dust extract induced lung epithelial cell model and treatment

Lung epithelial cells exposed to organic dust extract (ODE) exhibited clear evidence of stress and inflammatory response in a concentration- and time-dependent manner. Initial morphological observations under phase-contrast microscopy revealed cell shrinkage, rounding, and loss of adherence with increasing ODE concentration, particularly after 3 hours of exposure.

Experimental mouse model of ODE-induced lung inflammation

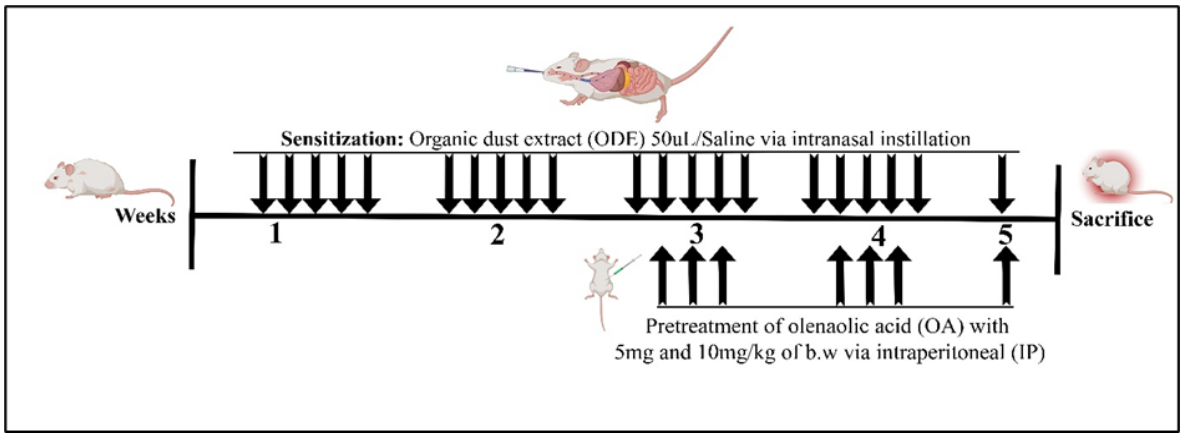


Figure-1: Experimental design for organic dust extract (ODE)-induced lung inflammation and oleanolic acid (OA) pretreatment in mice.

ODE treatment induces sPLA$_2$-IIA mRNA expression in lung epithelial cells

The delta Ct values revealed that the treating ODE of 0.25% significantly increased sPLA2-IIA mRNA expression in A549 lung epithelial cells, indicating an inflammatory response with fold increase of 4±0.1 folds. The sPLA$_2$-IIA mRNA expression in lung tissues of ODE group was significantly upregulated to 5.9-fold compared to the control group i.e., one-fold (Figure 2E). The findings suggested that exposure of animals to

organic dust extract trigger sPLA$_2$-IIA expression in lung epithelial cells and tissues.

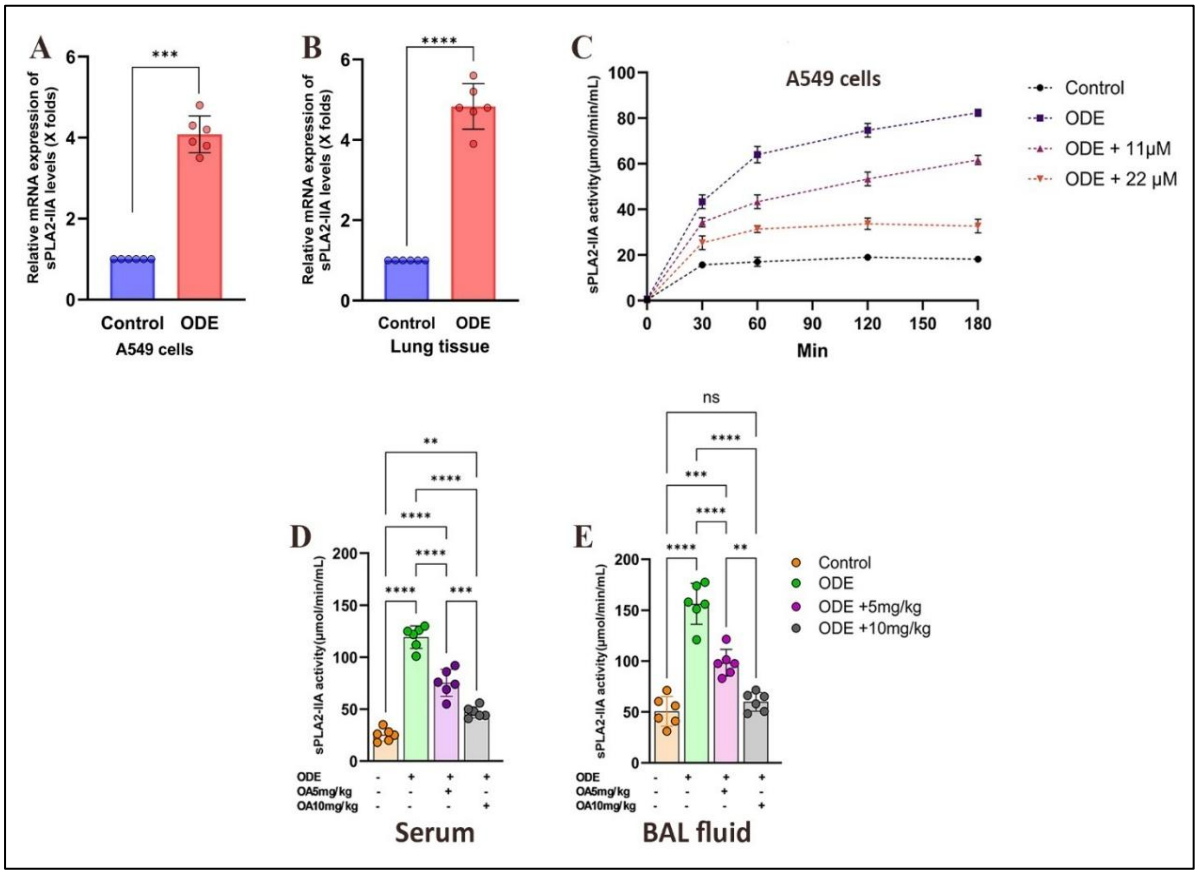


Figure 2: Quantification of sPLA$_2$-IIA expression and activity in ODE-sensitized and oleanolic acid-pretreated inflammatory models.

Oleanolic acid inhibits sPLA$_2$-IIA activity

Using oleanolic acid substantially reduced sPLA$_2$-IIA enzymatic activity in both animal and cellular models of ODE-induced inflammation. Exposure to ODE (0.25%) in A549 epithelial cells significantly boosted sPLA$_2$-IIA activity, reaching 61 ± 16 µM/min/mL compared to untreated controls (Figure 3.2C). Pretreatment with oleanolic acid dose-dependently suppressed enzyme activity in these cells. Oleanolic acid at 22 µM decreased sPLA$_2$-IIA activity by nearly half, reaching 24.6 ± 2 µM/min/mL in A549 cells. In vivo, repeated intranasal exposure to ODE increased sPLA$_2$-IIA activity in serum and BAL

fluid to 119.3 ± 4 and 156 ± 8 μM/min/mL, respectively (Figures 3.2D and 3.2E). Importantly, oleanolic acid pretreatment dramatically reduced this rise in a dose-dependent manner, with the 10 mg/kg dose suppressing enzyme activity to 47 ± 2 μM/min/mL in serum and 59.6 ± 2 μM/min/mL in BAL fluid. Oleanolic acid effectively inhibits $sPLA_2$-IIA activity in both in vitro and in vivo models, confirming its role as an effective inhibitor of ODE-induced inflammation.

Total and differential cell count of BALF

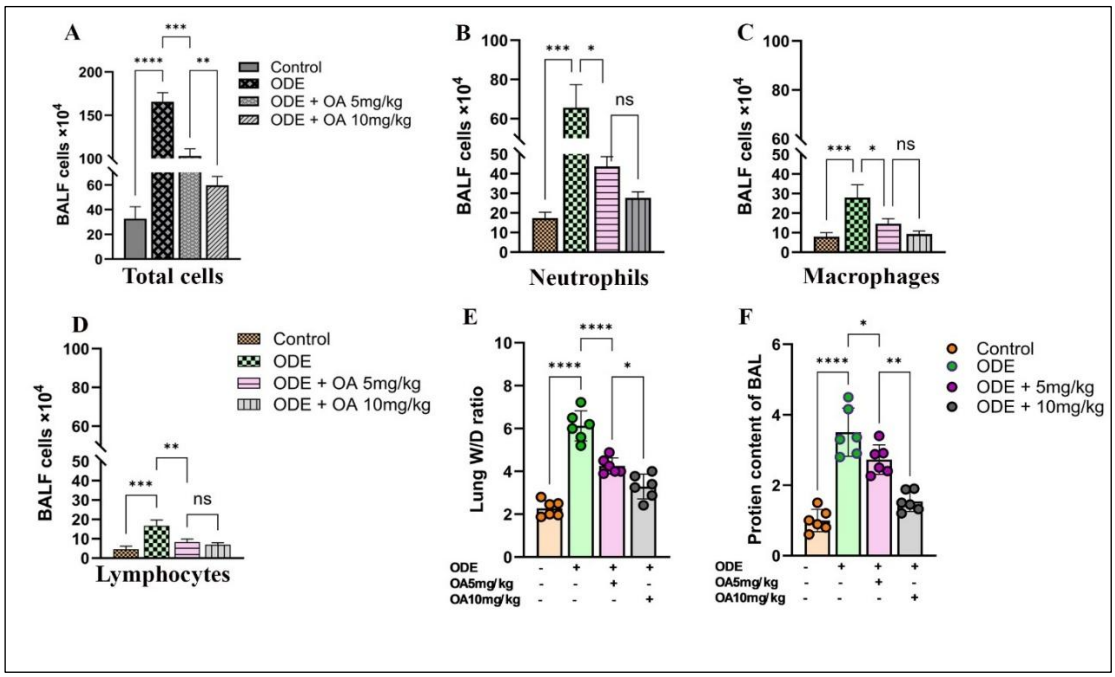


Figure 3: Effects of oleanolic acid (OA) on the cellular influx, edema, and total protein in lungs.

ODE-sensitized groups had increased total white blood cell (WBC) counts than controls, while oleanolic acid pretreatment reduced total cellular infiltration (Figure 3.3A). Differential analyses showed that ODE exposure caused immediate and chronic inflammatory responses by increasing neutrophils, macrophages, and lymphocytes. Oleanolic acid supplementation reduced neutrophil, macrophage, and lymphocyte recruitment.

Oleanolic acid modulates ODE induced histopathological changes in mouse lungs

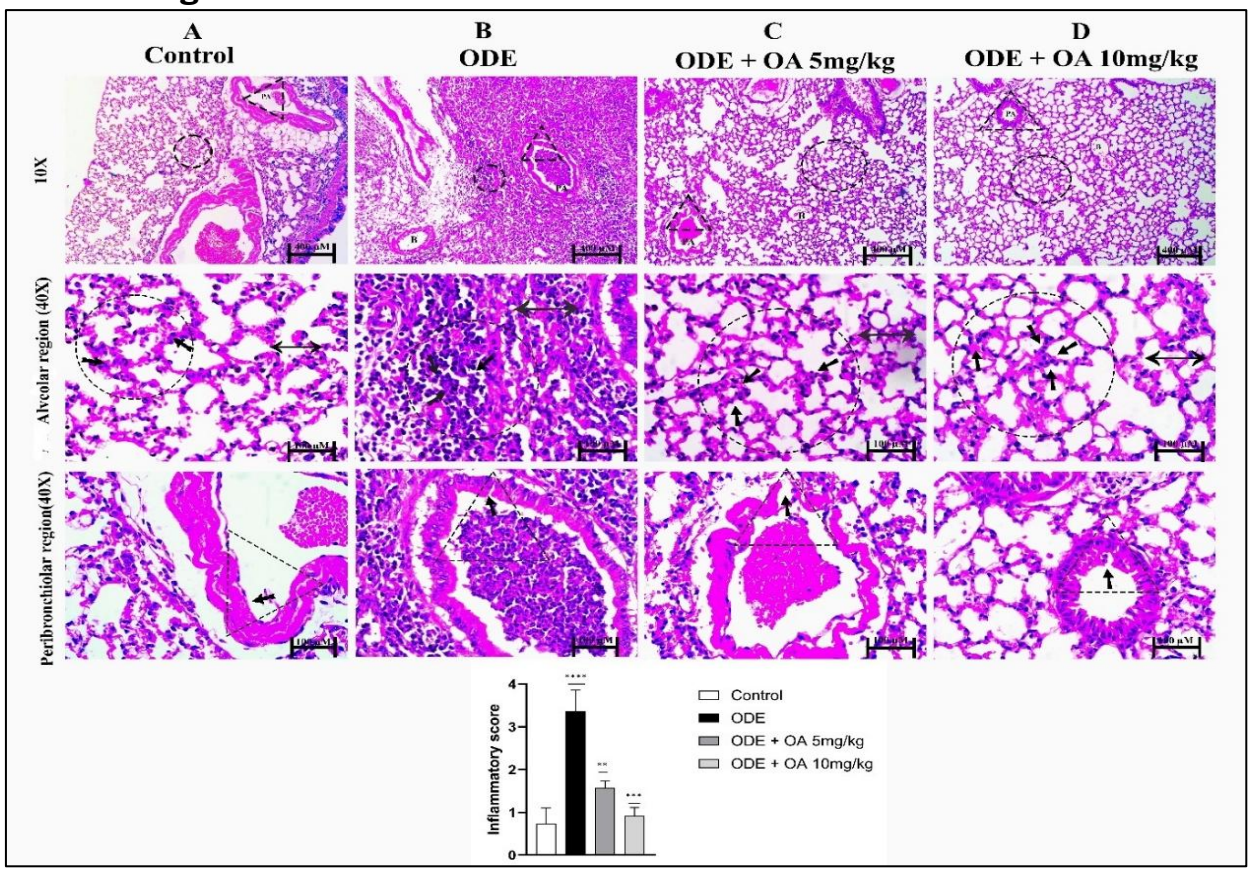


Figure 3.4: Histopathological evaluation of lung tissue following ODE exposure and oleanolic acid treatment.

Histopathological changes in ODE-sensitized mice's lung sections included inflammatory cell infiltration, bronchiolar epithelium disruption, and increased alveolar septal thickness. In ODE-exposed animals, quantitative scoring showed significantly enhanced pulmonary inflammatory indices, including 2.97±0.32 for alveolar infiltration, 2.33±0.1 for bronchiolar epithelial damage, and 3.35±0.49 for alveolar septal thickening. In contrast, pretreatment with oleanolic acid dose-dependently reduced alveolar infiltration scores, septal thickness, and epithelial sloughing.

Immunohistochemistry

IHC study examined the impact of oleanolic acid (OA) on $sPLA_2$-IIA expression in lung tissues after exposure to organic dust extract (ODE). Control animals showed low $sPLA_2$-IIA immunoreactivity, with no staining in alveolar epithelial cells (Figure 3.5A). In contrast, ODE-sensitized animals showed highly increased $sPLA_2$-IIA expression, mostly in bronchial epithelial cells, and significant inflammatory infiltrates (Figure 3.5B). A dose-dependent reduction in this reaction was caused by OA pretreatment. At 5 mg/kg, OA dramatically decreased $sPLA_2$-IIA immunostaining compared to the ODE group but still had greater expression levels than the control group ($p < 0.05$) (Figure 3.5C). A higher dose (10 mg/kg) of OA significantly reduced $sPLA_2$-IIA expression, practically recovering it to baseline levels (Figure 3.5D).

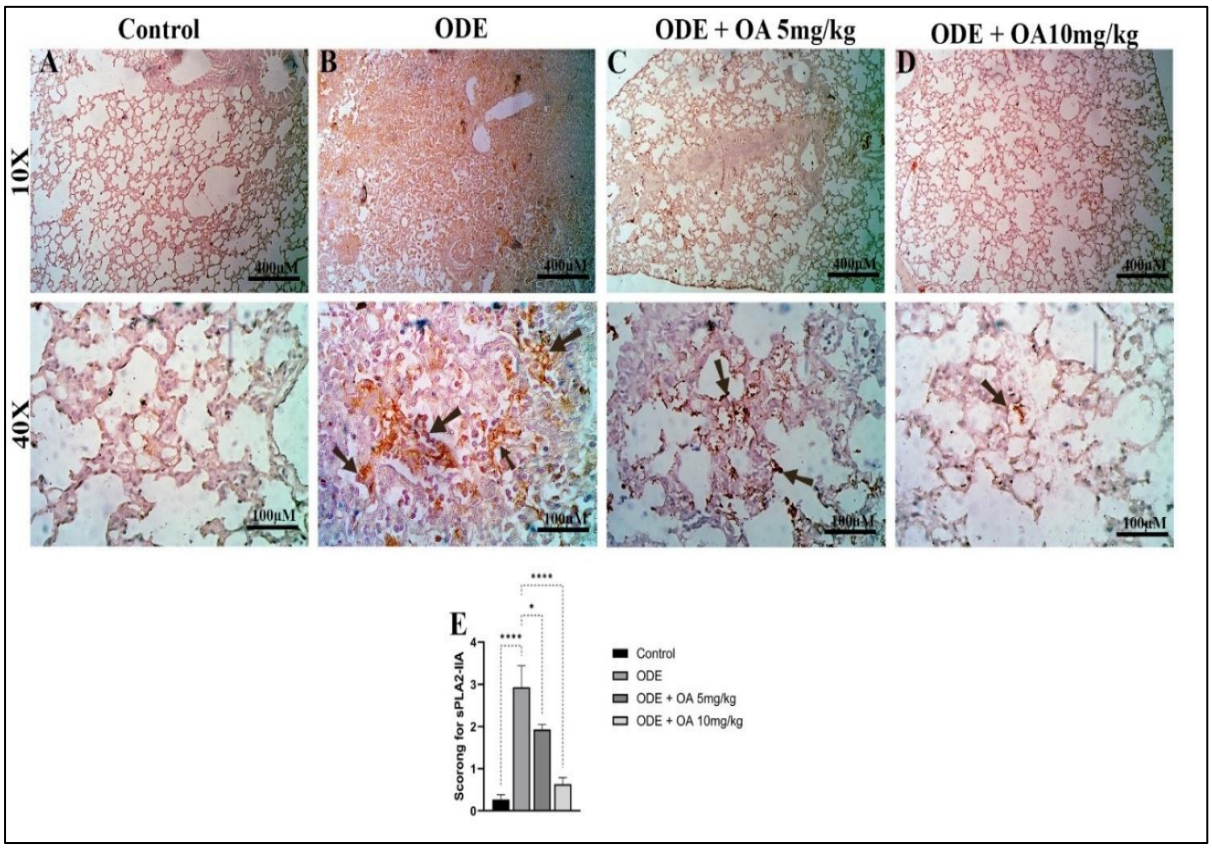


Figure 3.5. Immunohistochemical analysis of $sPLA_2$-IIA expression in lung tissues.

Inhibition of sPLA2-IIA enzyme attenuates activation of ERK1/2, P38 and cPLA$_2$

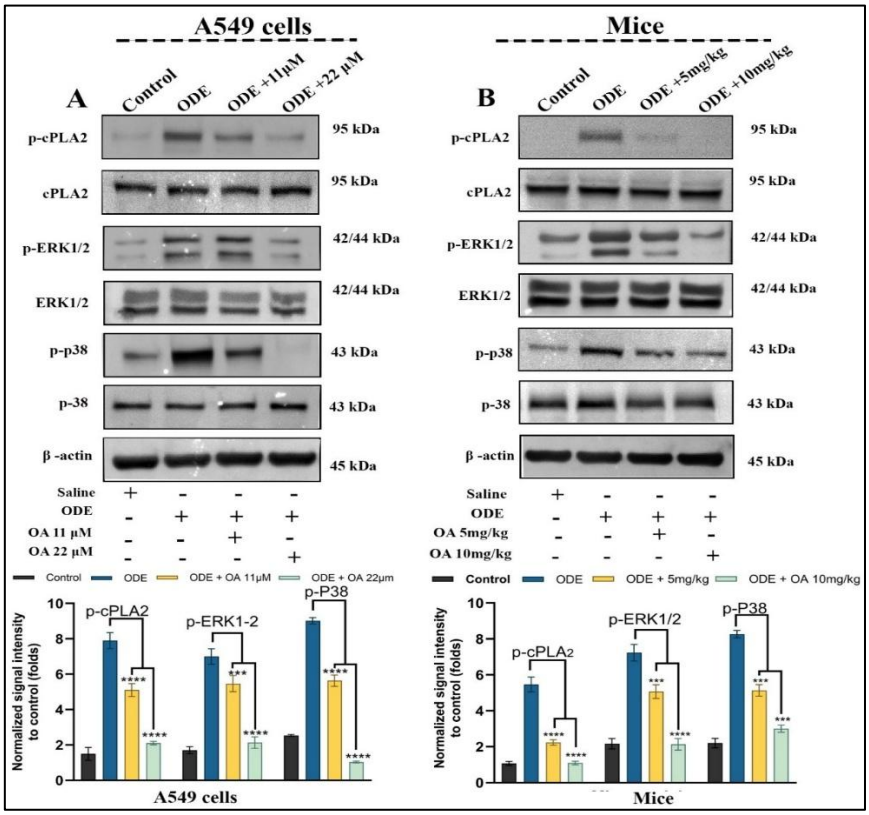


Figure 3.6: sPLA$_2$-IIA inhibition diminishes phosphorylation of ERK1/2, cPLA$_2$, and p38

The control group showed minimal baseline phosphorylation of ERK1/2Thr202/Tyr204, p38Thr180/Tyr182, and cPLA$_2$Ser505. In response to organic dust extract (ODE), A549 cells and lung tissue showed increased phosphorylation of ERK1/2, p38, and cPLA$_2$ (Figure 3.6A and 3.6B), indicating sPLA$_2$-IIA-mediated downstream signalling. A concentration-dependent reduction in phosphorylation events was caused by OA treatment. At lower concentrations, OA slightly reduced phosphorylation, but higher quantities significantly suppressed ERK1/2, p38, and cPLA$_2$ activation. Studies suggests that OA inhibits sPLA$_2$-IIA-driven MAPK signalling and cPLA$_2$ activation, disrupting a pro-inflammatory cascade produced by ODE stimulation.

Inhibition of sPLA$_2$-IIA mitigate COX-2 activity

Exposure of A549 lung epithelial cells to ODE significantly increased COX-2 activity, reaching 7.86 ± 0.6 U/mL in cell-based assays. OA treatment reduced COX-2 activity in A549 cells to 3.32 ± 0.3 U/mL, showing a concentration-dependent impact (Figure 3.7A). In the in vivo study, ODE-challenged animals showed increased COX-2 activity in BAL fluid (6.80 ± 0.6 U/mL), serum (8.38 ± 0.7 U/mL), and lung tissue homogenates (7.41 ± 0.4 U/mL) compared to the control group. Oleanolic acid dose-dependently inhibited COX-2 activity in all three compartments (Figure 3.7B–D). These findings indicate that OA inhibits COX-2 activity, reducing ODE-induced inflammation in cells and animals.

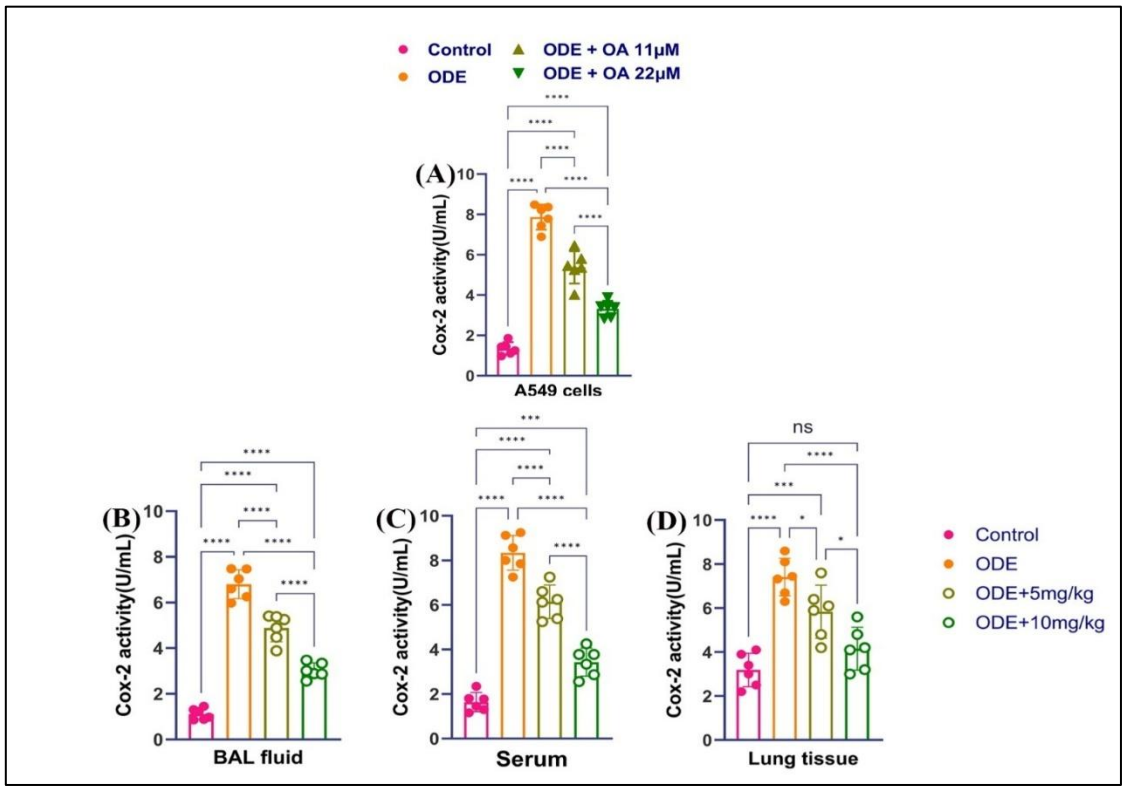


Figure 3.7: Effect of sPLA2-IIA inhibition on cyclooxygenase-2 (COX-2) activity.

Inhibition of sPLA2IIA modulates the secretion of pro-inflammatory cytokines and chemokines

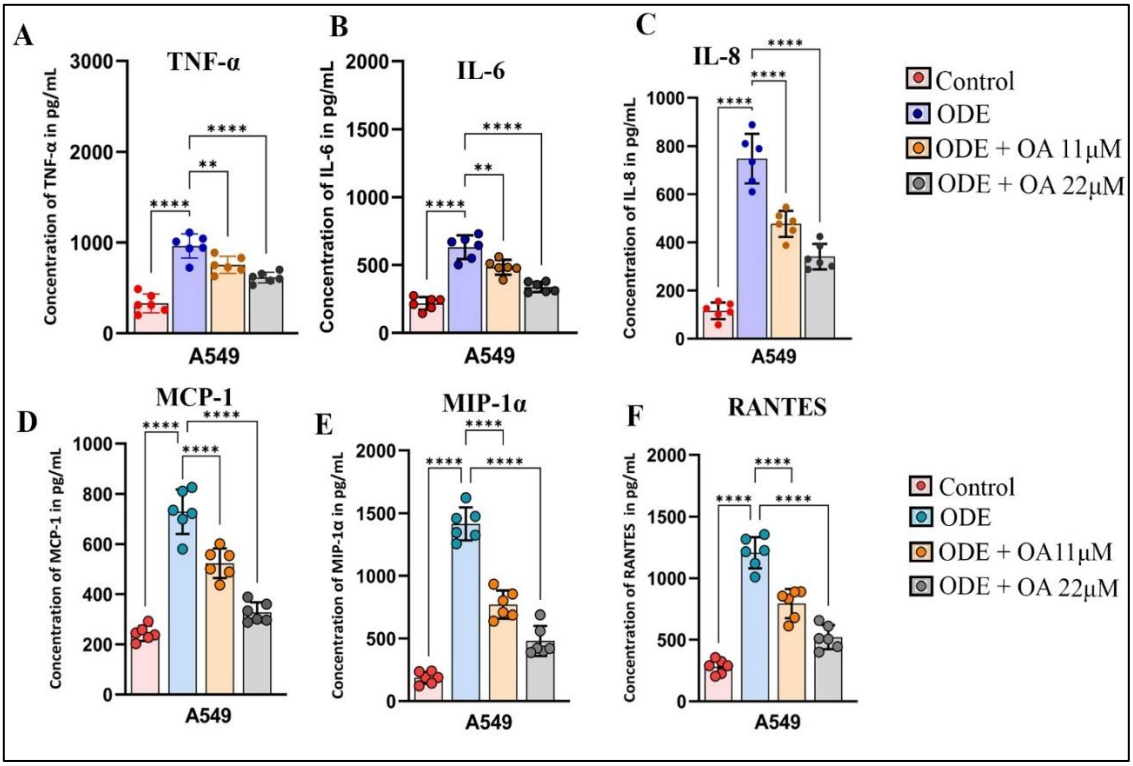


Figure 3.8: The effect of sPLA$_2$-IIA inhibition on secretion of cytokines and chemokines in A549 lung epithelial cells

To understand how sPLA2-IIA regulates inflammatory mediators, we examined cytokine (IL-6, TNF-α, and IL-8) and chemokine (MCP-1/CCL2, MIP-1α/CCL3, and RANTES/CCL5) secretion in in vitro and in vivo models. Exposure to ODE significantly increased the release of pro-inflammatory cytokines TNF-α, IL-6, and IL-8 in A549 lung epithelial cells compared to control groups (Fig. 3.8A–C). These cytokines were also raised in BAL fluid, serum, and lung tissue homogenates from ODE-exposed mice (Fig. 3.9A–C), indicating a significant inflammatory response. The effect of ODE on chemokine secretion was also examined. Both A549 cells and ODE-challenged animals showed elevated levels of MCP-1/CCL2, MIP-1α/CCL3, and

RANTES/CCL5, which are associated with immune cell recruitment and lung inflammation (Fig. 3.8D–F; Fig. 3.9D–F).

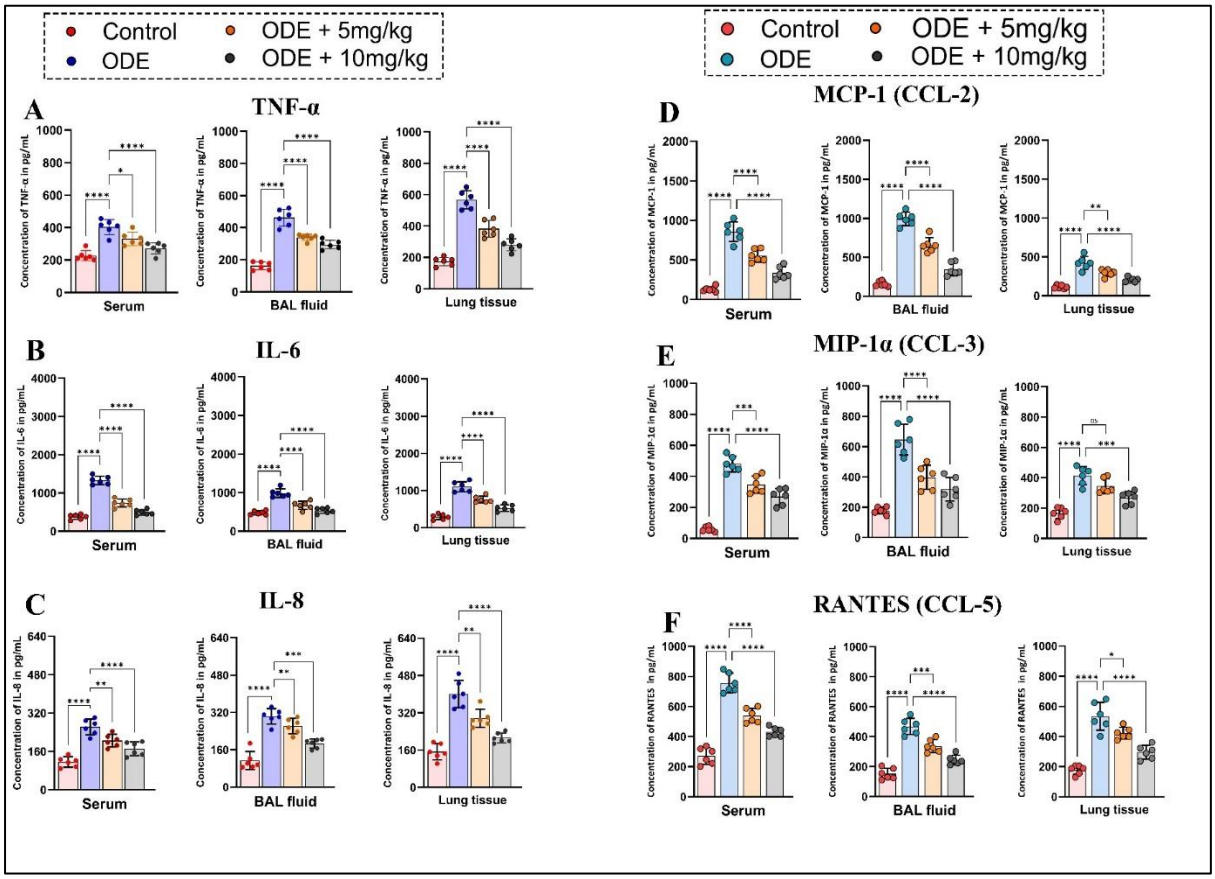


Figure 3.9: The effect of sPLA$_2$-IIA inhibition on secretion of cytokines and chemokines in mice model

sPLA2-IIA inhibition mitigates the PGE-2 & TXB2 production

After treatment to ODE in A549 cells (Figure 3.10A), PGE-2 levels rose from 133 ± 16.9 pg/mL (control) to 805 ± 43.3 pg/mL in the ODE group. In A549 cells, oleanolic acid (22 μM) dramatically reduced PGE-2 secretion to 260 ± 15.8 pg/mL, indicating a dose-dependent effect of sPLA2-IIA inhibition on PGE-2 synthesis. Similar trends were seen in the in vivo mouse model. Control animals had baseline PGE-2 levels of 505 ± 59.9 pg/mL in BAL fluid, 450 ± 32.9 in serum, and 280 ± 30 in lung tissue homogenates. Chronic ODE exposure caused a substantial increase in levels to 2968 ± 155 pg/mL in BAL

fluid, 3857 ± 171 in serum, and 1460 ± 138 in lung tissue homogenates (Figure 3.10B–D). Treatment with oleanolic acid (10 mg) significantly reduced PGE-2 levels, resulting in 916.5 ± 76.5 pg/mL in BAL fluid, 752 ± 66.2 in serum, and 504 ± 63 in lung tissue homogenates.

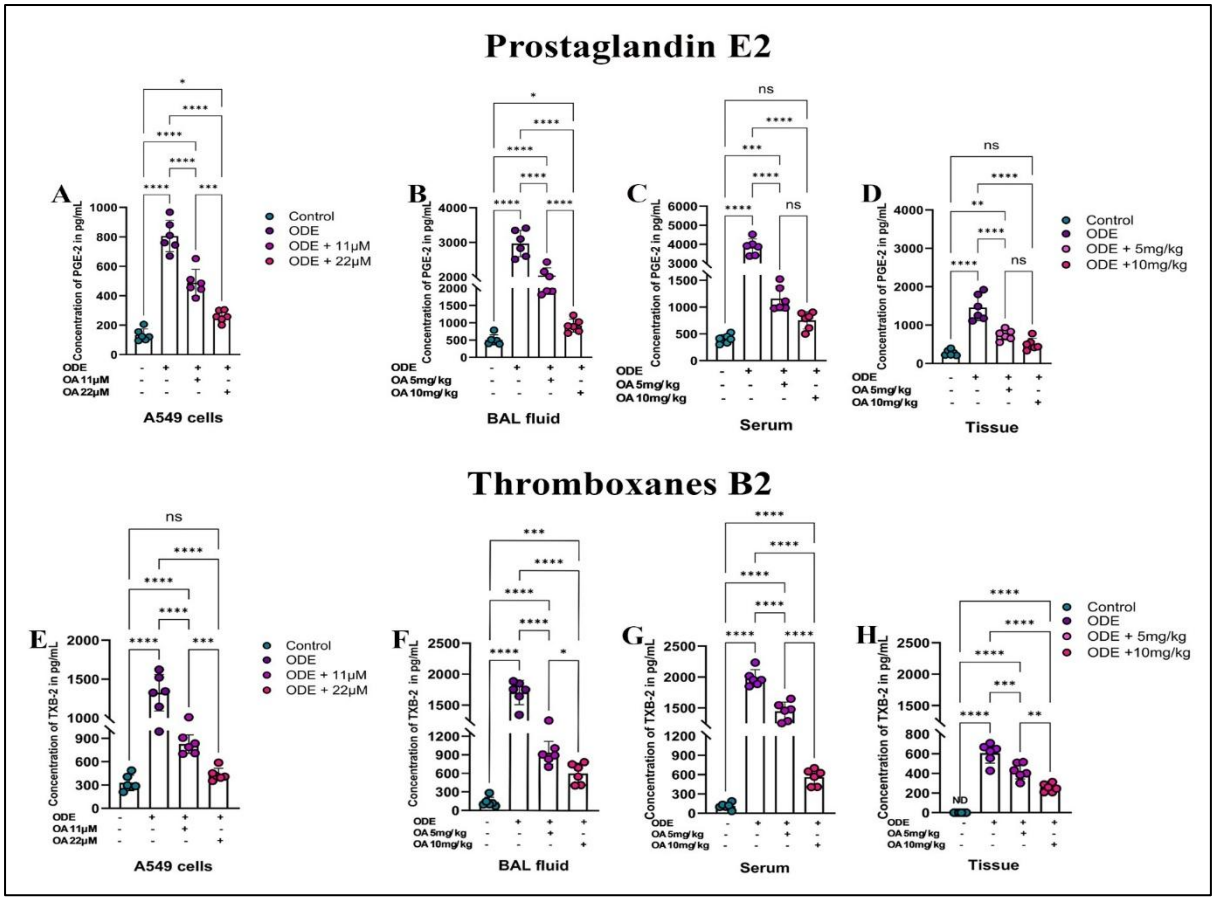


Figure 3.10: sPLA2-IIA inhibition modulates prostaglandin E2 (PGE-2) and thromboxane B2 (TXB2) synthesis in ODE-induced lung inflammation.

DISCUSSION

Dust from agriculture and animals can cause respiratory illnesses. Chronic inflammation from repeated dust exposures may cause these disorders. Bacterial TLR ligands, extracellular vesicles, and proteases in organic dust cause lung inflammation (Poole et al., 2012). The TLRs, ROS, and MAPKs signalling cascade via NFkB and Stat3 induces lung inflammation. ERN1, an ER stress-UPR sensor, regulates organic dust-induced lung inflammation (Kusampudi et al., 2025). Lipid signalling molecules are well-known inflammatory mediators, but their significance in organic dust-induced lung inflammation is unclear.

This study found that poultry organic dust extracts elevated sPLA2-IIA mRNA expression and enzymatic activity in A549 lung epithelial cells and mouse lungs for the first time. Alveolar-capillary barrier disruption enhanced epithelial and vascular permeability, which increased BAL fluid protein and sPLA2-IIA activity (Chen et al., 2009). Arachidonic acid, released from the hydrolysis of membrane phospholipids by sPLA2-IIA, is a precursor for bioactive lipid mediators like prostaglandins and leukotrienes, which modulate vascular tone and permeability and leak plasma proteins into alveolar space (Balsinde and Balboa, 2005), explaining the increased protein levels in BAL fluid in mice repeatedly exposed to ODE. sPLA2-IIA suppression by oleanolic acid reduced BAL protein level, indicating its role in ODE-induced pulmonary oedema. Lysophosphatidic acid, prostaglandins, and leukotrienes, sPLA2-IIA products, signal inflammatory responses and chemotaxis, resulting in immunological and inflammatory cell infiltration (Murakami et al., 2017).

ODE enhanced lung infiltration of neutrophils, macrophages, and lymphocytes, whereas oleanolic acid reduced it, highlighting the importance of sPLA2-IIA. Oleanolic acid irreversibly inhibits secretory phospholipase A2, which causes its anti-inflammatory effects (Dharmappa et al., 2009). Along with its anti-inflammatory capabilities, oleanolic acid improved the histological appearance of ODE-treated lung tissue. ODE increased chemokine and cytokine secretion in A549 lung epithelial cells and mice, which was inhibited by oleanolic acid, indicating increased sPLA2-IIA expression and activity, explaining its critical role in regulating inflammatory cytokine and chemokine induction.

The cellular and molecular mechanisms by which sPLA2-IIA modulates inflammatory mediator induction and responses have not been studied. ODE treatment elevated phosphorylation of cPLA2, ERK, and p38 MAPKs, which was blocked by oleanolic acid, showing that activation of these pathways induces inflammatory mediator production and secretion. Besides enzymatic function, sPLA2 regulates host defence and signal transduction (Murakami et al., 2017). In response to inflammation, sPLA2-IIA binds to integrins αvβ3 and αvβ1 to activate membrane-bound M-type receptors (PLA2R1) and initiate intracellular signalling, activating ERK and P38 MAPKs through phosphorylation (Fujita et al., 2014; Saegusa et al., 2008). Calcium increases (Kramer and Sharp, 1997) and ERK and p38 MAPKs phosphorylate cPLA2 Ser505 to activate enzymes and produce arachidonic acid (Lin et al., 1993). Poultry ODE and trypsin isolated from it elevate intracellular calcium and promote inflammatory mediator expression and secretion in Beas2B lung

epithelial cells (Kusampudi et al., 2023). NADPH oxidase activation by intracellular calcium increases ROS generation. In rat and lung epithelial cells, poultry ODE activates NOX enzymes to modulate inflammatory mediator levels (Meganathan et al., 2022).

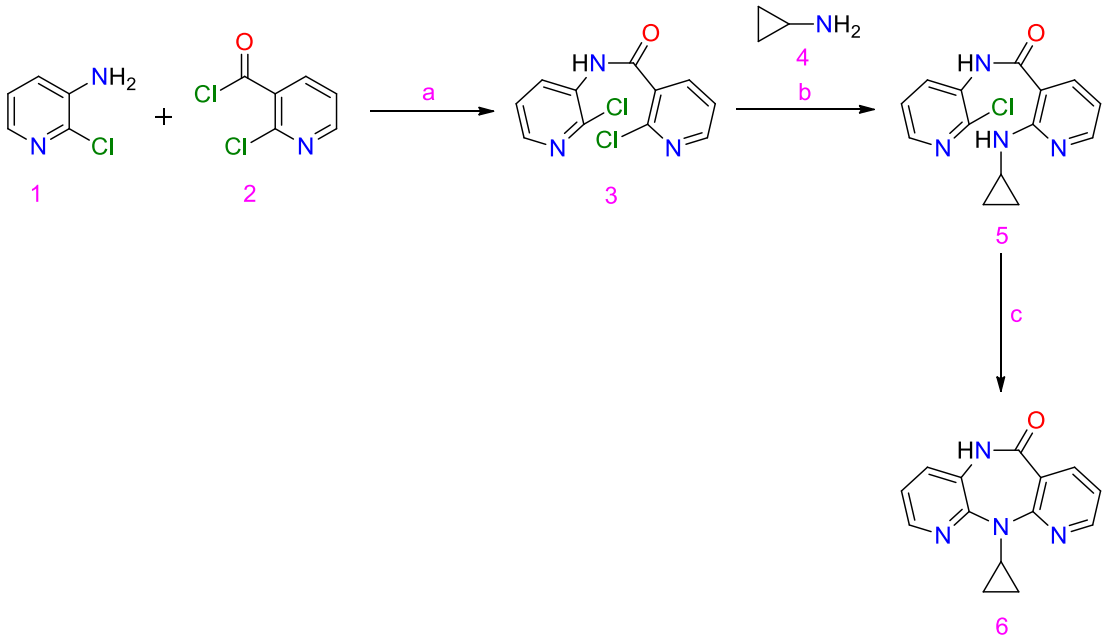
ODE activation of cPLA2 increases intracellular arachidonic acid levels for conversion to prostaglandins, leukotrienes, and thromboxanes by cyclooxygenases, lipoxygenases, and cytochrome P450 enzymes (Wang et al., 2021). ODE increased COX-2 activity, PGE2, and TXB2 levels in A549 lung epithelial cells, mouse lung, BALF, and serum. Oleanolic acid prevented these increases, highlighting sPLA2's regulatory role. Increased ROS and lipid mediators may increase cytokines and chemokines, worsening inflammation.

CONCLUSION

Our investigations showed that sPLA2 mediated signalling through ERK and p38 MAPKs and that cPLA2 regulates ODE-induced lung inflammation, making it a therapeutic target for agricultural and animal farm workers' occupational lung illnesses. A549 bronchial epithelial cells are airway-specific, however cell characteristics and proliferation can affect experimental repeatability. The present study's ODE concentration may not adequately reflect chicken farm workers' ODE exposure. At higher doses, oleanolic acid may have off-target effects that were not examined in this study. Future investigations to understand ODE control of sPLA2-IIA gene expression and identify ODE components responsibl

RESULTS

Scheme: Synthesis of *11-cyclopropyl-5H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6(11H)-one* 6.



Our previous studies have shown that the tricyclic dipyrido diazipinone exhibits anti-inflammatory properties by inhibiting both human and snake venom sPLA$_2$-IIA in vitro and in vivo models (Thimmegowda et al., 2007). In the present study, the in-silico, in vitro, and in vivo studies have been conducted to determine the efficacy of tricyclic dipyrido diazipinone derivative 6 (f) towards poultry dust induced lung inflammation.

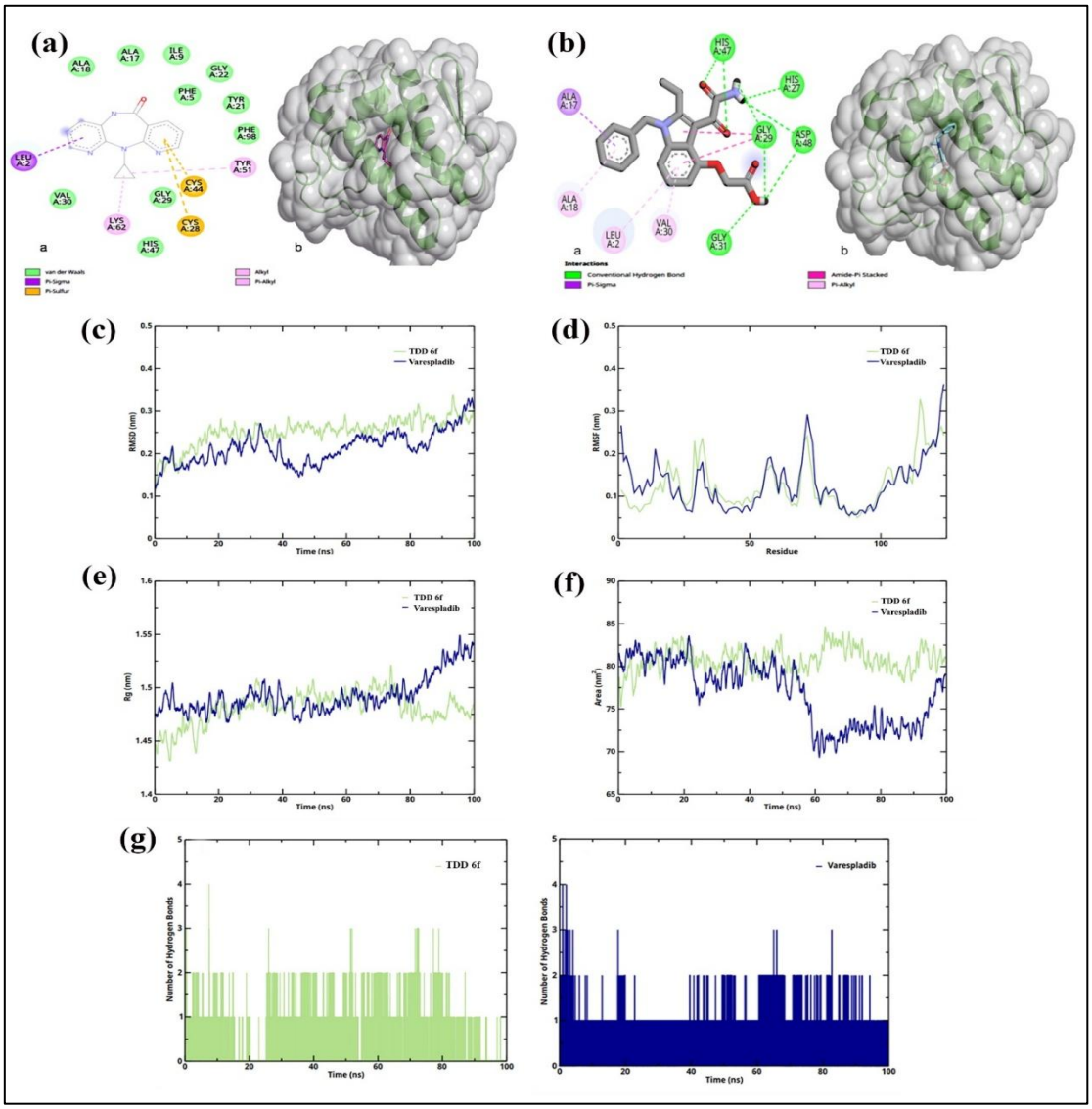


Figure 4.1: *In silico* analysis of sPLA$_2$-IIA inhibitor TDD 6f

11-cyclopropyl-5H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6(11H)-one 6 (TDD 6f) demonstrated superior binding affinity and structural stability compared to the standard inhibitor varespladib during in silico docking and molecular dynamics simulations. The TDD 6f exhibited stronger interactions with critical protein residues, stable RMSD, compact Rg, and consistent hydrogen bonding. Additionally, it showed enhanced adaptability and hydrophobic stability with reduced conformational flexibility. These qualities

demonstrate TDD 6f's therapeutic potential and applicability for improvements in sPLA$_2$-IIA-mediated inflammatory pathways.

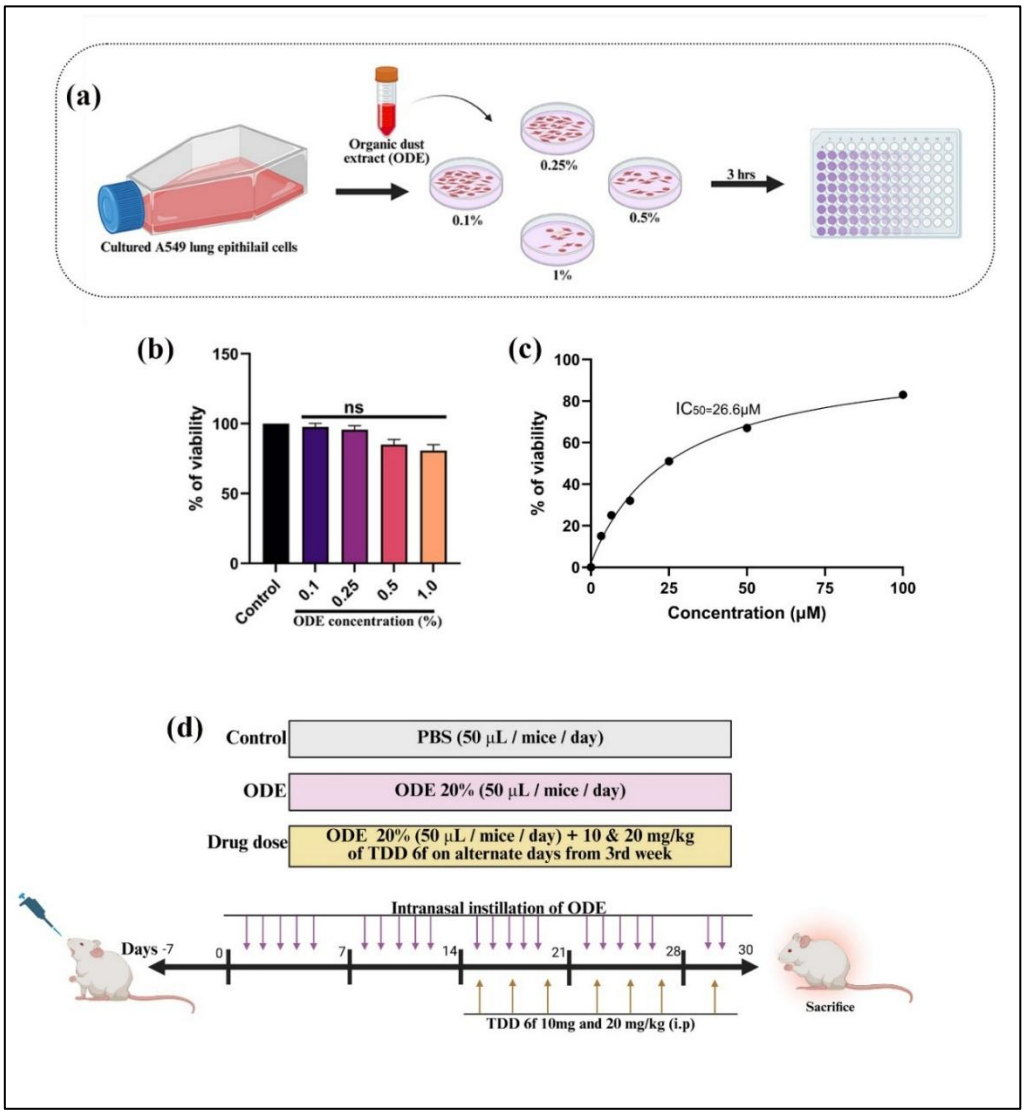


Figure 4.2: Experimental design to study the effects of poultry Organic Dust Extract (ODE) and Tricyclic Dipyrido Diazepinone 6f (TDD 6f) on lung inflammation in A549 lung epithelial cells and mice

Human lung epithelial A549 cells were treated with poultry dust extract (0.1% to 1%) for 3 hours (Figure 4.2a) to develop an in vitro inflammatory model. Cell shrinkage, detachment from culture plates, and loss of a cobblestone-like morphology occurred. At 0.25% dust concentration, epithelial cells responded optimally to inflammation (Figure 4.2b). To test if TDD 6f (13.3 and 26.6 μM) decreases dust-induced inflammatory responses, parallel experimental

groups were treated 1 hour before ODE administration (Figure 4.2c). TDD 6f reduced human lung epithelial A549 cell inflammation substantially. ODE (1%, 5%, 12.5%, 20%, and 25%) was administered to mice according to the protocol (Miller et al., 2002; Poole et al., 2009), and their weight was measured weekly. At 20% ODE, mice showed strong inflammatory responses without weight change. ODE-induced inflammation was dose-dependently decreased by the sPLA2-IIA inhibitor TDD 6f (10 and 20 mg/kg body weight). Finally, animals were euthanised, and BAL fluid, blood serum, and lung tissue were collected for inflammatory biomarker analysis (Figure 4.2d).

Organic dust extract increases sPLA$_2$-IIA expression and enzyme activity

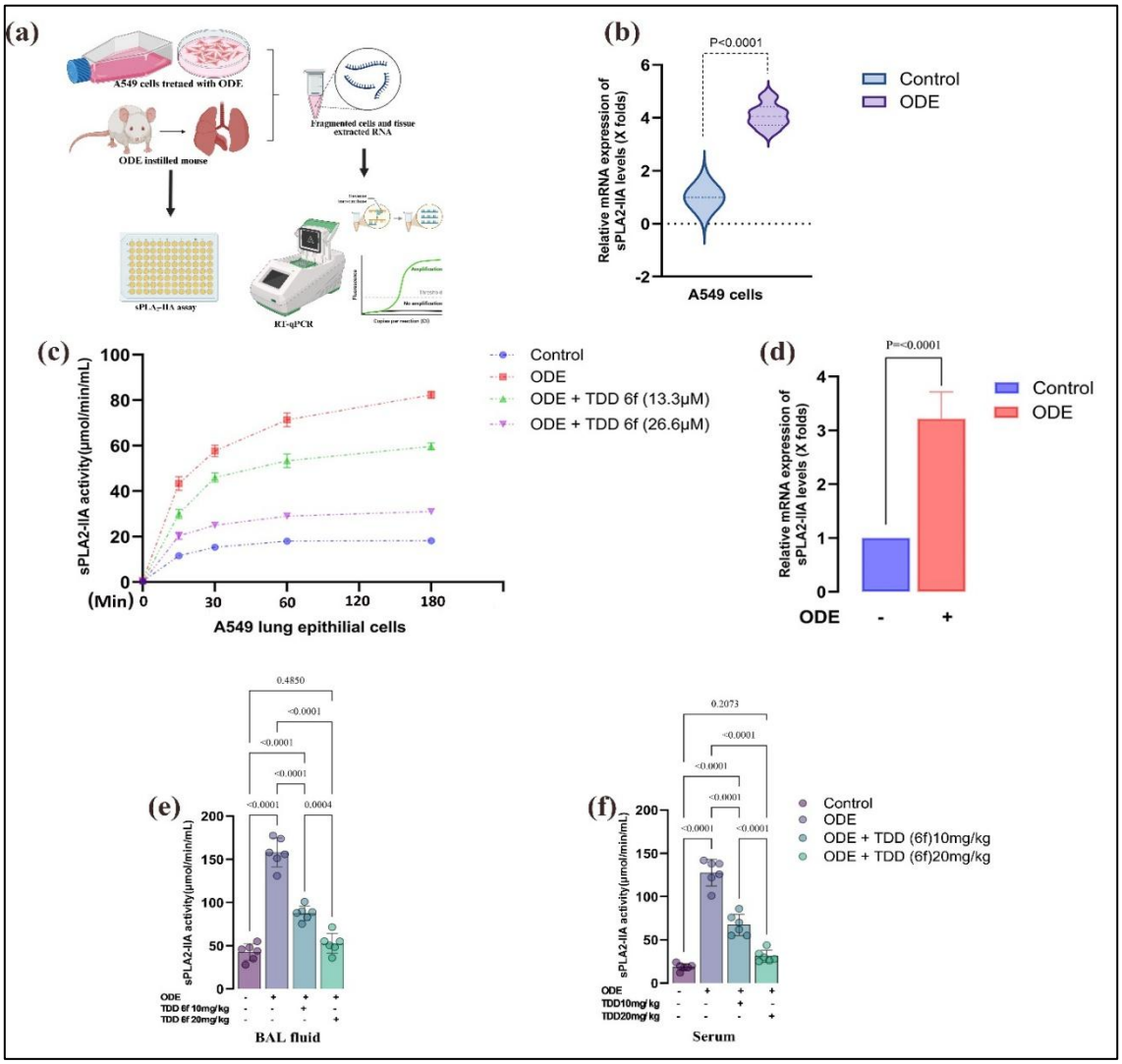


Figure 4.3: Quantification of sPLA$_2$-IIA mRNA expression and enzyme activity

In A549 lung epithelial cells, ODE treatment led to a 4.08 ± 0.8 fold increase in sPLA2-IIA mRNA expression (P<0.0001) (Figure 4.3b) and an increase in sPLA2-IIA activity (82.54 ± 4 μM/min/mL vs. 18 ±1.6 μM/min/mL). Using TDD 6f, sPLA2-IIA activity in A549 cells was lowered to 31 ± 2 μM/min/mL (Figure 4.3c).

In mice treated with ODE, sPLA2-IIA expression in lung tissue increased by 3.21 ± 0.8-fold compared to the control (1-fold) (P < 0.0001) (Figure 4.3d). BAL fluid (157.9 ± 6.8 μM/min/mL) and serum (127.8 ± 6 μM/min/mL) showed increased enzyme activity compared to controls (Figure 4.3e and 4.3f). The administration of TDD 6f decreased sPLA2-IIA activity in both BAL fluid (52.75 ± 4.7 μM/min/mL) and serum (31.17 ± 2.8 μM/min/mL These findings showed that ODE treatment increased A549 cell and mouse lung sPLA2-IIA expression and activity.

Inhibition of sPLA$_2$-IIA reduces cellular influx, total protein and edema

Total leukocyte count was used to determine sPLA2-IIA inhibition's effect on lung inflammation. Instilled ODE mice had a greater leukocyte count than untreated control mice (mean ± SEM:168 ± 10.8×104 vs 21.33 ± 1.7×104, P<0.001). Using the selective sPLA2-IIA inhibitor TDD 6f, mice showed a substantial decrease in total leukocytes (60.33 ± 2.9×104 cells, P<0.001) (Figure 4.4a). Inhibiting sPLA$_2$-IIA significantly reduces the recruitment of inflammatory cells. In the differential cell count, ODE therapy boosted neutrophils to 54.67 ± 3.7×104, while TDD6f decreased them to 21.67 ± 2×104. Overall macrophages and lymphocytes decreased slightly (Figure 4.4b-d). Inflammatory cell infiltration and sPLA$_2$-IIA expression were

positively linked (R^2= 0.92, p< 0.01), supporting the concept that this factor is crucial for cellular infiltration.

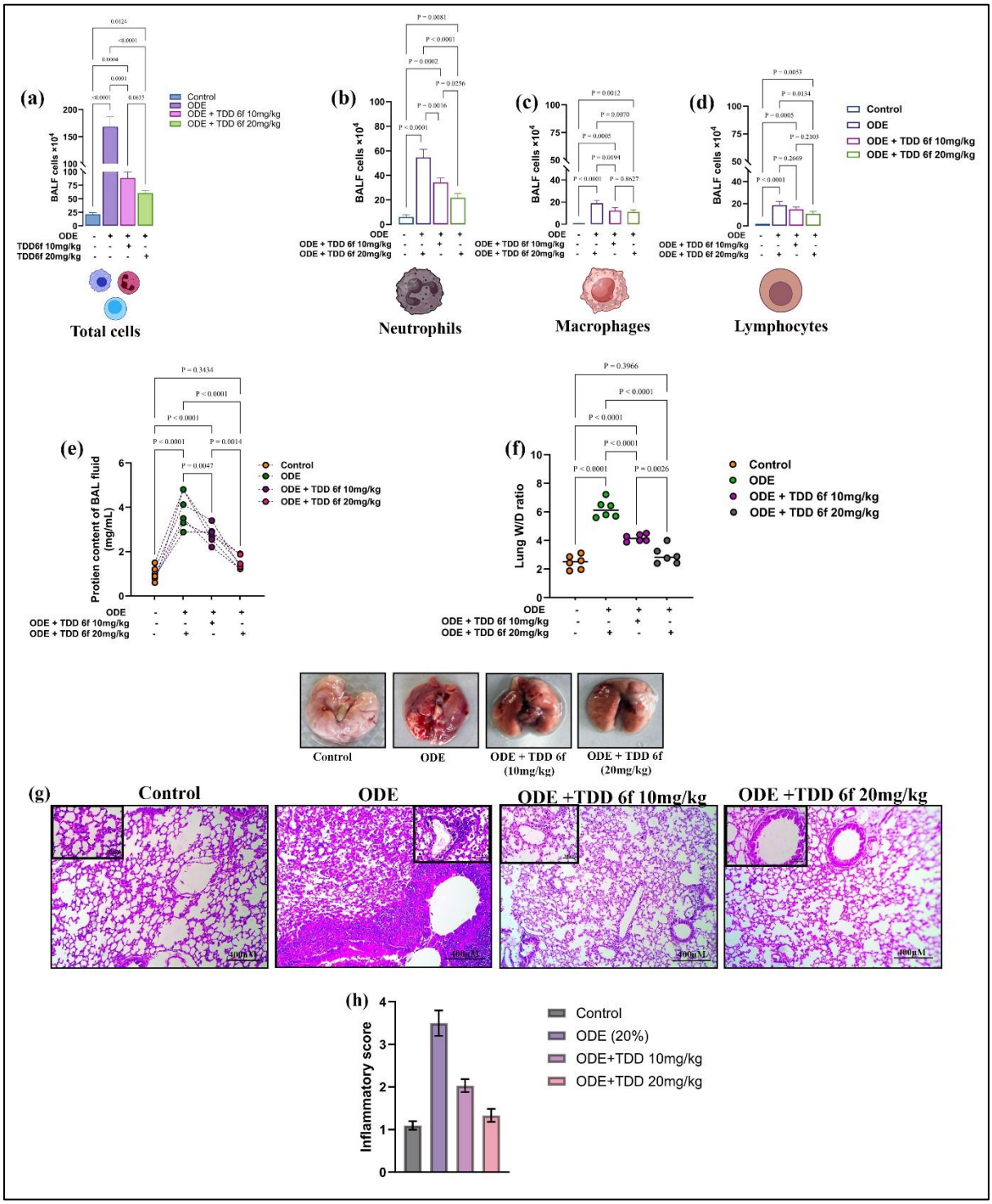


Figure 4.4: Effects of sPLA$_2$-IIA inhibition on the cellular influx, edema, total protein in lungs, and histopathology

The study found that mice treated with ODE had higher levels of total protein in their BAL fluid (3.9 ± 0.6mg/mL), indicating increased permeability of the alveolar-capillary barrier. The sPLA2-IIA inhibitor TDD 6f significantly decreased total protein concentration by 1.49 ± 0.1mg/mL compared to ODE treatment ($P<0.01$) (Figure 4.4e). Mice given ODE had a higher lung wet-to-dry (W/D) ratio (6.21 ± 0.5) compared to the control group (2.44 ± 0.4) receiving PBS. Furthermore, treatment TDD 6f reduced pulmonary oedema to 2.9 ± 0.2 (Figure 4.4f). These data confirm TDD 6f's anti-inflammatory effectiveness.

TDD 6f treatment mitigates histopathological alterations in pulmonary tissue

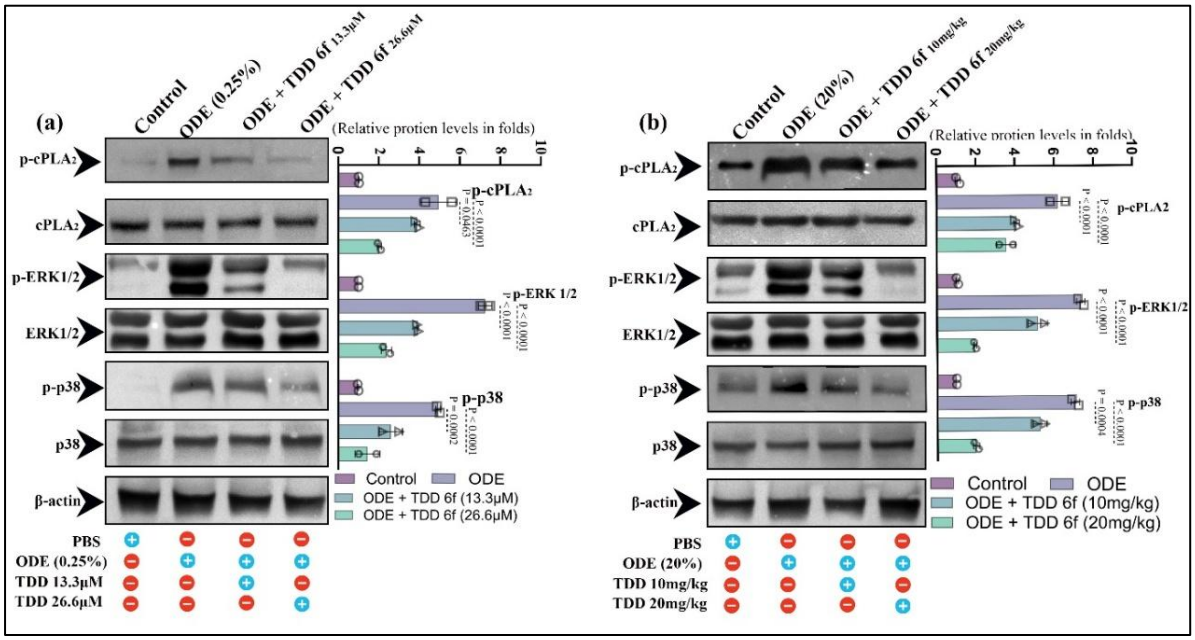


Figure 4.5: Effects of TDD 6f on sPLA$_2$-IIA mediated phosphorylation of ERK1/2, p38, and cPLA$_2$

To study how TDD 6f affects downstream signalling pathway activation by sPLA$_2$-IIA, we measured MAP kinases (ERK1/2 and p38) and cPLA$_2$ phosphorylation in A549 cells and animals. Protein lysates from A549 cells and lung tissue were analysed for ERK1/2, p38, and cPLA$_2$ activation using

phospho-specific antibodies. ODE treatment in A549 cells and mice elevated ERK1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), and cPLA2 (Ser505) phosphorylation, indicating MAPK pathway activation. A549 cells and mice treated with the sPLA2-IIA inhibitor TDD 6f demonstrated a consistent decrease in ERK1/2, p38, and cPLA2 phosphorylation, suggesting downregulation of the signalling pathway.

Inhibition of $sPLA_2$-IIA diminishes the proinflammatory cytokines and chemokines

Previous experiments revealed that dust extract treatment in lung epithelial A549 cells and mice increased cPLA2 activation, leading to increased levels of cytokines (IL-6, IL-8, TNF-α, MCP-1, MIP-1α, and RANTES) (Figures 4.6a-d, 4.6e-h, 4.6i-l). The sPLA2-IIA inhibitor therapy dramatically reduced cytokines and chemokines ($p<0.001$), indicating downstream signalling inhibition. Inhibition of secretory phospholipase A2 significantly reduces proinflammatory cytokines and chemokines, suggesting its importance in the inflammatory response.

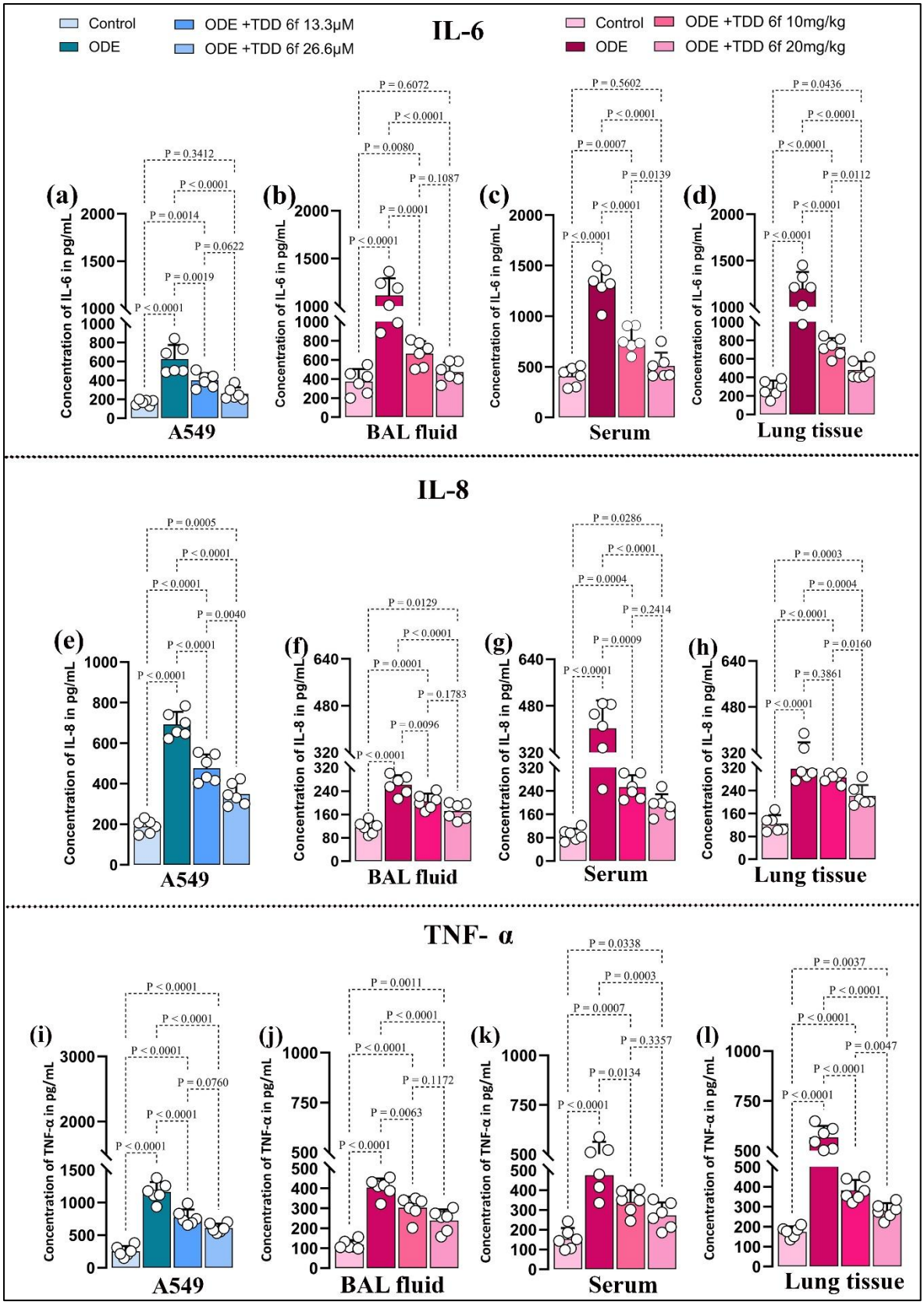


Figure 4.6: The effect of sPLA$_2$-IIA inhibition on the secretion of cytokines in A549 lung epithelial cells and mice

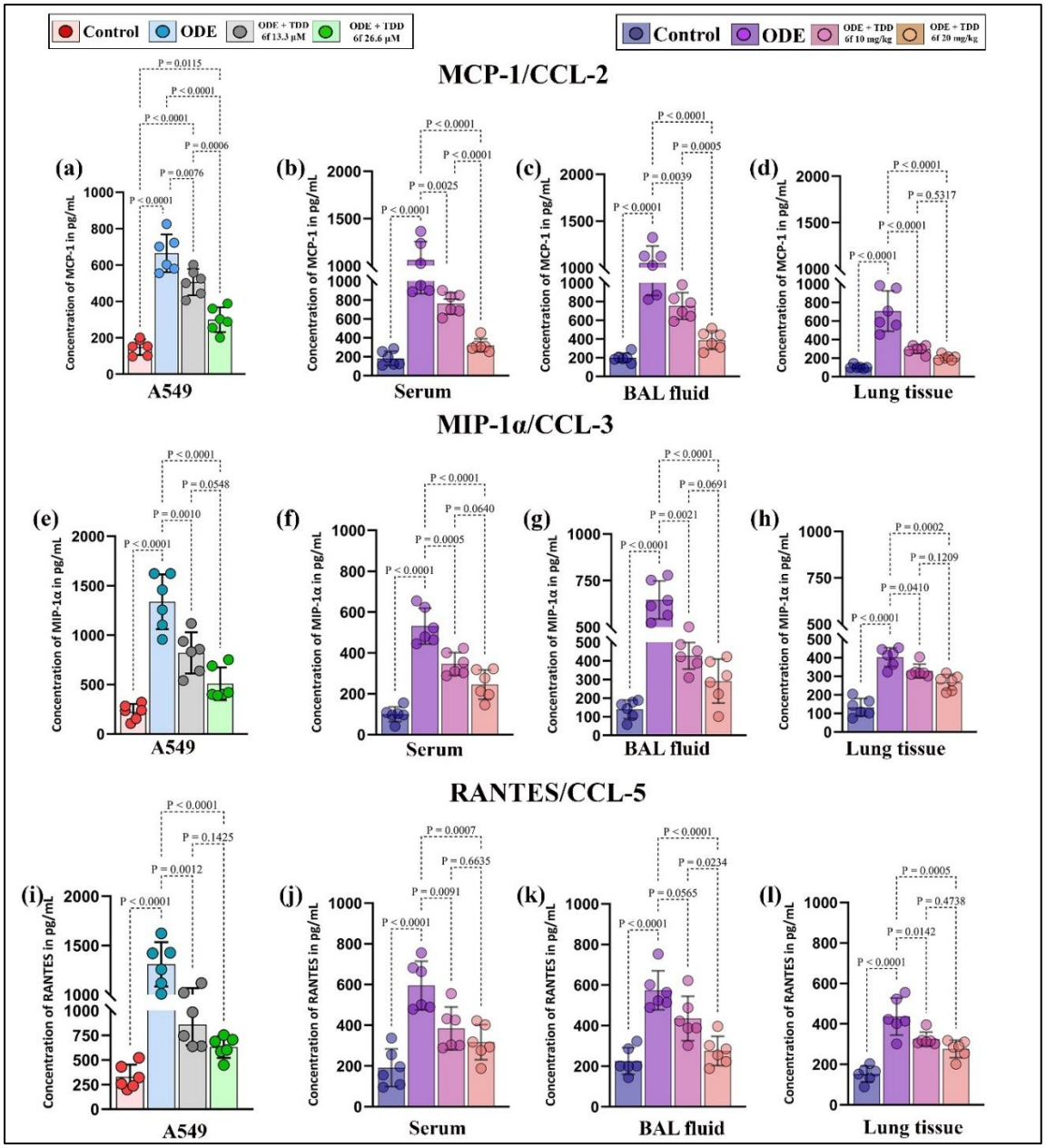


Figure 4.7: The effect of sPLA$_2$-IIA inhibition on the secretion of chemokines in A549 lung epithelial cells and mice.

Inhibition of sPLA$_2$-IIA by TDD 6f dampens the COX-2 activity

A549 cells pretreated with sPLA2-IIA inhibitor (TDD 6f) had significantly reduced COX-2 activity (Figure 4.8b). Mice given 20% ODE had increased COX-2 activity than controls in vivo. BAL fluid, serum, and lung tissue COX-2 activity was significantly reduced in mice co-treated with the sPLA2-IIA inhibitor TDD 6f (Figure 4.8c-e). Our study found that TDD 6f reduces sPLA$_2$-

IIA activity, which inhibits COX-2 activity, suggesting an effective therapeutic approach for pulmonary inflammatory conditions.

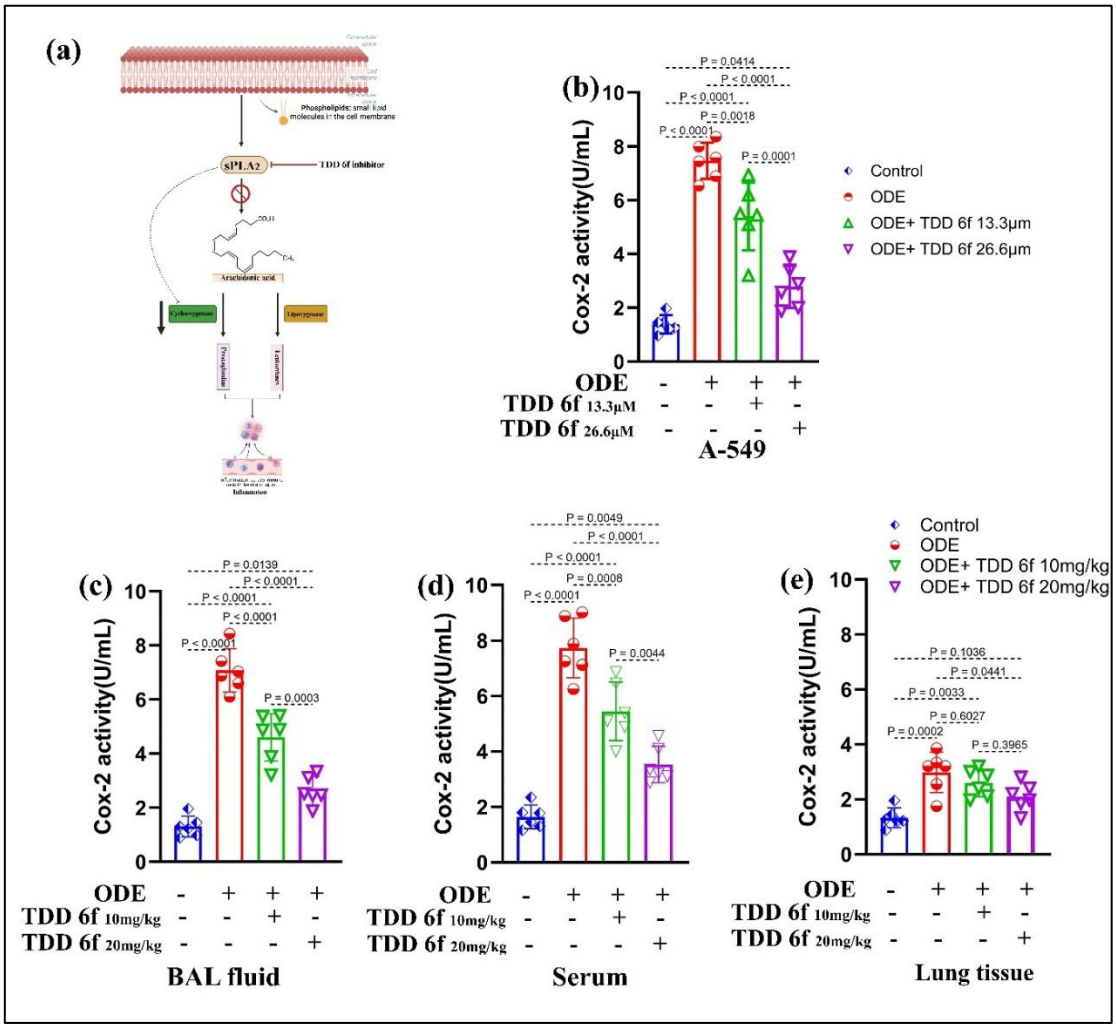


Figure 4.8: Inhibition of sPLA$_2$-IIA reduces cyclooxygenase-2 (COX-2) activity in A549 cells and mice.

TDD 6f decrease the sPLA$_2$-IIA mediated PGE2 synthesis

A549 cells treated with ODE showed a substantial increase in PGE2 levels ($p<0.0001$), associated with increased sPLA$_2$-IIA expression and COX-2 activity, indicating that sPLA2 influences downstream PGE-2 secretion. TDD 6f (13.3 µM and 26.6µM) pretreatment of A549 cells showed dose-dependent

suppression of PGE-2 levels, indicating the inhibitory effects of sPLA2-IIA on PGE-2 levels (Figure 4.9b).

In the in vivo model, mice given 20% ODE had higher PGE-2 levels in bronchoalveolar lavage fluid, serum, and lung tissue homogenate than controls. Mechanistic findings showed that intraperitoneal sPLA2-IIA inhibitor TDD 6f dose-dependently decreased PGE-2 levels (Figure 4.9c-e). The results indicate that inhibiting sPLA2-IIA dramatically lowers PGE2 synthesis and the proinflammatory response at both cellular and systemic levels.

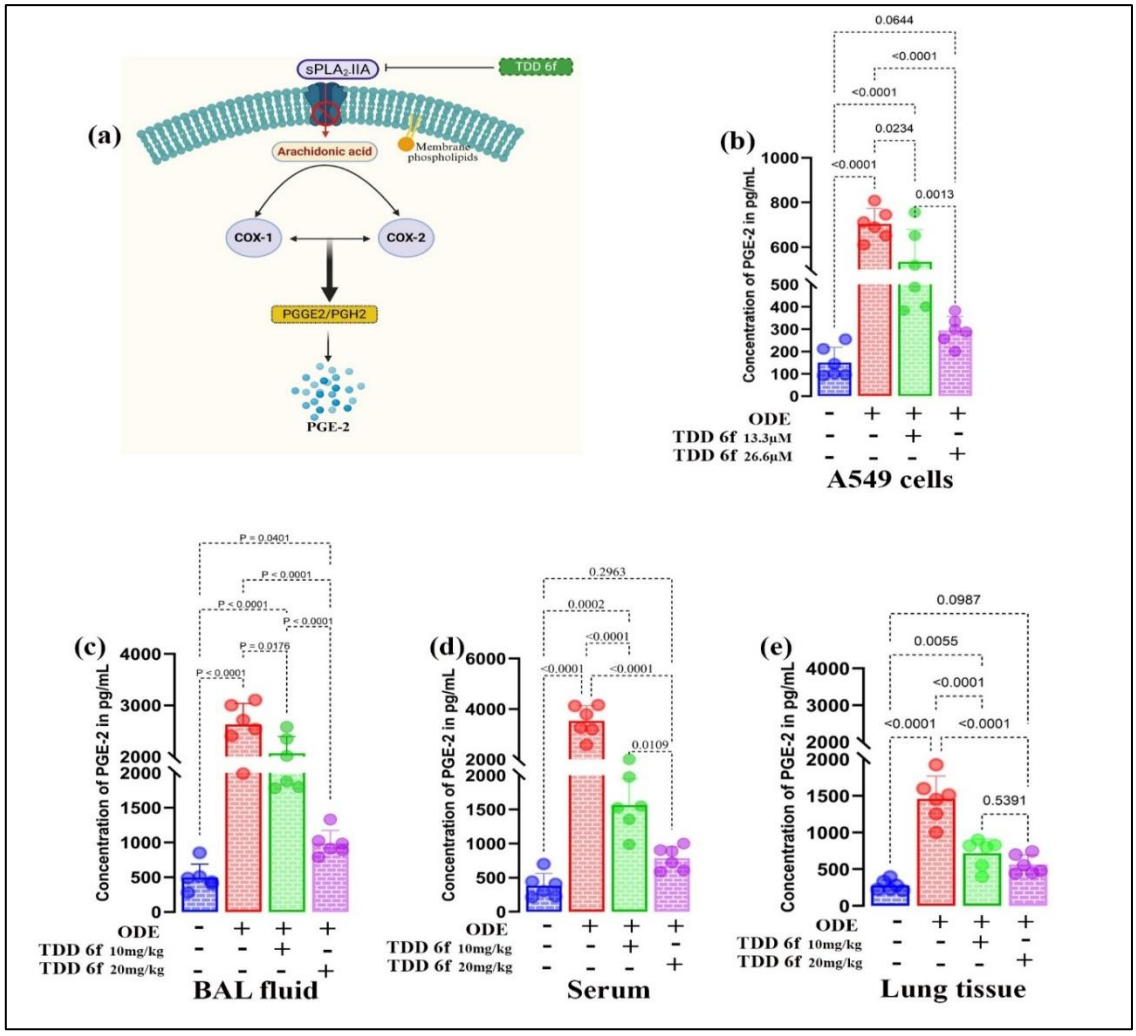


Figure 9: Effect of sPLA$_2$-IIA inhibition on prostaglandin E2 (PGE2) production in lung epithelial cells and mice

sPLA$_2$-IIA inhibitor TDD 6f reduces inflammation in the lungs by limiting the levels of TXB$_2$

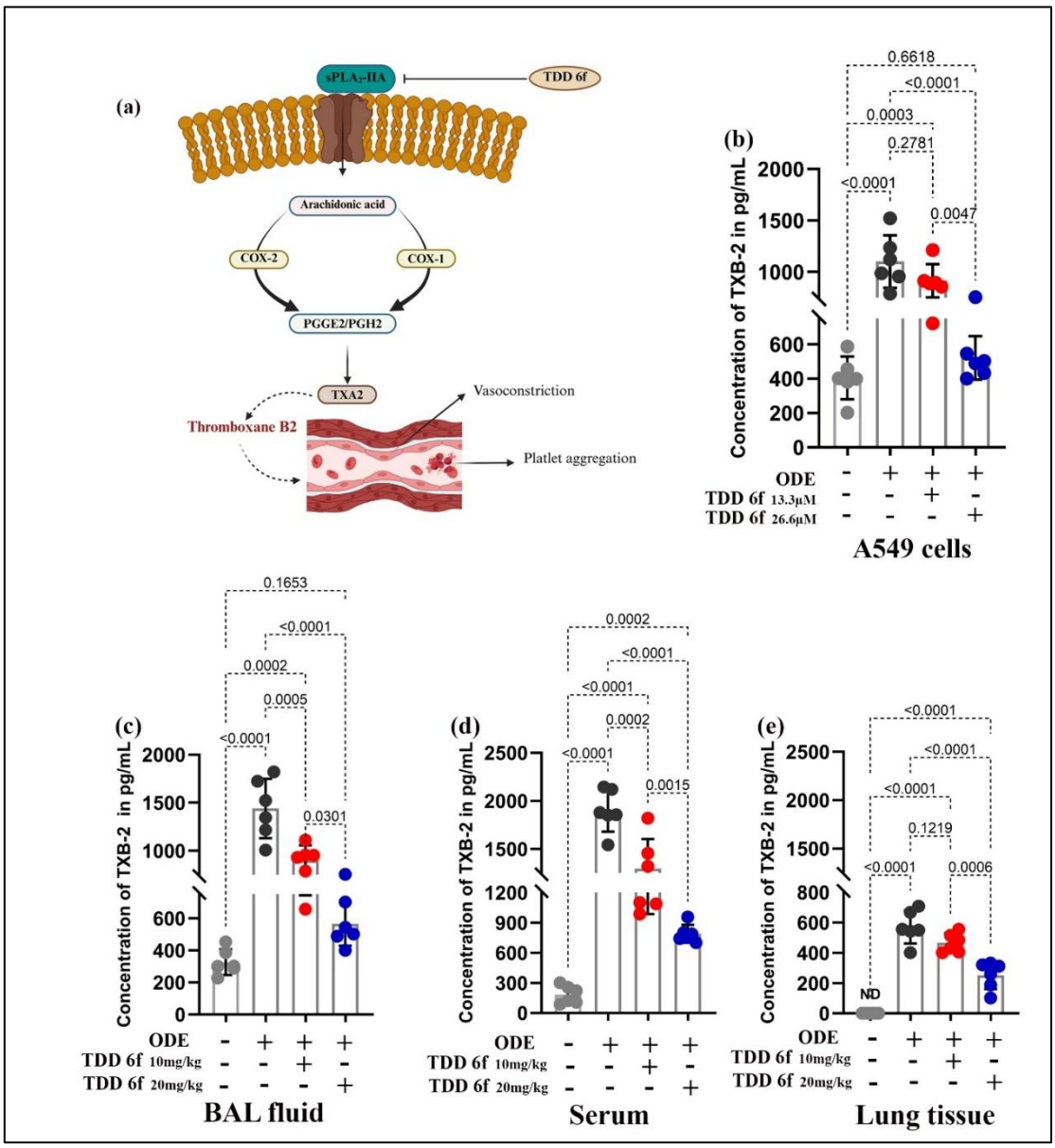


Figure 4.10: TDD 6f lowers the TXB-2 level in A549 lung epithelial cells and mice.

The ODE-stimulated A549 lung epithelial cells had increased TXB2 levels than the untreated control ($p<0.0001$). Pretreatment with TDD 6f suppressed secretory phospholipase A2 group IIA enzyme activity, reducing TXB2 dose-dependently in A549 cells (Figure 4.10b).

In vivo validation confirms *in vitro* results that TDD 6f attenuates the downstream arachidonic acid pathway, lowering TXB-2 levels in mice's BAL fluid, serum, and lung tissue). The results showed that TDD 6f inhibits sPLA2-IIA to change proinflammatory mediator synthesis.

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PAPERS PRESENTED IN CONFERENCES

1. Presented a POSTER presentation & Participated in the International Conference **on "*Biotechnology* as a Catalyst for Sustainable Development (*HEAL*-BioTec 2023)"** organized by the Department of Biotechnology and Bioinformatics, School of Life Sciences, JSS Academy of Higher Education and Research, Mysore, India on 7th to 9th December 2023.

   Title: - ***"Oleanolic acid, a putative sPLA$_2$ enzyme inhibitor, supresses organic dust induced lung inflammation by downregulating sPLA$_2$-IIA"***

2. *Presented an oral presentation & Participated in International Conference **on "Recent Advancements in Chemistry"** organized by Department of Chemistry, Mangalore University FMKMC college, Madikeri, Karnataka, India on 23rd November 2022.
   ***Best Oral Presentation**

3. Presented an oral presentation at **International Conference on Sustainable utilization of Bioresources** at Department of Botany, University of Kerala. India in 10-15th January 2022

   Title: - ***"Role of sPLA$_2$ in lung inflammatory disorders of poultry farm workers"***

LIST OF PUBLICATIONS

From the thesis:

1. **Deepadarshan Urs,** Kiran B.K, Prabhakar B.T, Giresha A.S, Pushpavathi H.R, Shankar J, Sathisha A.D, Vijay Boggaram, Dharmappa K.K. Organic dust induces lung inflammatory responses via sPLA 2 -IIA mediated activation of cPLA 2 and MAP kinases by enhancing the production of eicosanoid lipid mediators (**Under review**)

2. **Deepadarshan Urs, Abdul Rahman, H.S Shuba, N.D Satyanarayan, Rajkumar S. Meti, K.K Dharmappa**· sPLA2-IIA inhibitor Tricyclic Dipyrido Diazepinone derivative 6f suppresses poultry organic dust-induced pulmonary inflammation by downregulating ERK1/2-cPLA2 pathway. *Biomedicine and Pharmacotherapy* (**Accepted**)

Apart from the thesis:

1. Vani Karadi Manjappa, Manjula Mavathuru Venkatappa, **Deepadarshan Urs**, Suliphuldevara Mathada Basavarajaiah, et al., Urolithin-C Suppresses Inflammation by Blocking NF-kB Signaling Pathway in LPS-Induced RAW 264.7 Macrophages. *Journal of Inflammation Research.*2025 Aug 18:1-21.

2. Al Amin M, Bouhenni H, Zehravi M, Sweilam SH, Durgawale TP, Qureshi MS, Durgapal S, Haque MA, Vodeti R, **Deepadarshan Urs**, Shatu MM. Natural compounds and programmed necrosis: pioneering a new frontier in cancer treatments. Naunyn-Schmiedeberg's Archives of Pharmacology. 2025 Mar 26:1-27.

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6. **Deepadarshan Urs,** M. Narayanappa, P. Sophiya, H. Krishnaram, S. N. Pramod, Dharmappa KK. Medicinal and nutritional aspects of wild edible fruits from Western Ghat of India. *International Journal of Green Pharmacy (IJGP)*;16(4) (2023).

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BOOK CHAPTERS

1. **Deepadarshan Urs**, Giresha A.S, Tejaswini H.K, Prabhakar B.T, Dharmappa K.K. "Natural Bioactives from the Endophytes of Medicinal Plants" *Edible foliage endophytes and their natural metabolites: Significance in Supporting Human Health* (2024)

2. **Deepadarshan Urs**, Anil Madesh, Karrar Mahmood, Nagaraja Sreeharsha, and K. K. Dharmappa. "Real-time utilization of nanostructured biosensors for the determination of food toxins." *Novel Nanostructured Materials for Electrochemical Bio-sensing Applications* (2023): 367.

3. Sophiya, P., **Deepadarshan Urs,** Rajkumar S. Meti, HA Nagma Banu, J. Shankar, and K. K. Dharmappa. "Voltammetric Devices for Disease Detection." *Voltammetry for Sensing Applications* (2022): 339.

Acknowledgements

I would like to thank for providing JRF fellowship from Department of Science and Technology - Science and Engineering Research Board (DST-SERB), Government of India, under project No. EEQ/2019/000456, and an SRF fellowship from Mangalore University. I indebted to the Department of Studies in Biochemistry, Mangalore University PG centre, Kodagu & Molecular Biomedicine Laboratory, Sahyadri Science college, Shivamogga for providing an opportunity to carry out this research work.

Signature of Research Scholar Research Supervisor